Spatial and Temporal Accumulation of Defense Gene Transcripts in Bean (*Phaseolus vulgaris*) Leaves in Relation to Bacteria-Induced Hypersensitive Cell Death

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Primary leaves of 7- to 9-day-old Red Mexican bean plants were inoculated with virulent or avirulent isolates of *Pseudomonas syringae* pv. *phaseolicola*, or saprophytic *P. fluorescens* either by vacuum infiltration of the whole leaf lamina, or by syringe-inoculation of selected leaf panels. In the incompatible combination, resistance was associated with a hypersensitive response (HR). Syringe-inoculated leaves were sampled in three zones: zone 1, the inoculated leaf area; zone 2, the surrounding 0.5–0.7 cm of leaf tissue; and zone 3, the remainder of the leaf. Northern blots of RNA from zones 1, 2, and 3 were probed with bean cDNAs for phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), chitinase (CHT), and lipoxygenase (LOX). Accumulation of PAL, CHS, and CHT transcripts was more rapid and generally of greater magnitude in the incompatible than in the compatible interaction and, in both cases, was observed essentially only in zone 1 tissues. Similarly, antibacterial phytoalexins were only detected in zone 1 from the incompatible interaction. Young primary leaves have a background level of LOX transcripts, which declines as leaves age. This decline was accelerated over the first 12 hr postinoculation (hpi) with avirulent bacteria, whereas a weak transient induction, peaking at 5–6 hpi, was observed in the compatible interaction. A subsequent, strong accumulation of LOX transcripts was seen in both the compatible and incompatible interactions outside the inoculation site starting about 14 hpi. LOX transcripts did not accumulate at the inoculation site itself in the incompatible interaction compared to a relatively strong induction in the compatible interaction. Interestingly, inoculation of leaves with cells of the saprophyte *P. fluorescens* also induced the accumulation of transcripts for CHS, CHT, and LOX, but generally to a lesser degree than in the incompatible interaction. No HR occurred and no macroscopic cell damage was apparent in leaves inoculated with *P. fluorescens*. However, at the microscopic level individual, trypan blue-stained, necrotic plant cells were visible. In spite of this and the accumulation of CHS transcripts, no phytoalexin accumulation was found up to 48 hr after inoculation. The spatial and temporal relationship of the hypersensitive reaction to defense gene transcript and phytoalexin accumulation is discussed.

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Plants respond to inoculation with avirulent pathogens with a variety of putative defense responses such as the accumulation of antimicrobial phytoalexins (Dixon 1986); hydrolytic enzymes, e.g., chitinase and β-1,3-glucanase (Boller 1987); callose deposition (Kauss 1987); lignification (Friend 1976); and hypersensitive cell death (Klement 1963, 1982; Lyon and Wood 1976). Many of these responses are regulated, at least in part, at the level of transcription (reviewed in Collinge and Slusarenko 1987; Dixon and Lamb 1990).

The hypersensitive response is a descriptive name for the rapid necrosis that host tissues undergo when challenged with avirulent or nonhost pathogen isolates. The designation "rapid" is in relation to the slower appearance of necrosis in disease development in the compatible interaction. It appears that the host cells are not being killed directly by some toxic principle from the pathogen, but that the host cells are activated to undergo a kind of programmed cell death that depends on host protein synthesis (Sequeira 1976; Keen *et al.* 1981; Lyon and Wood 1977). The precise way in which host cells die is not yet clear, but mechanisms that damage membranes, either by generation of active oxygen species or more direct enzymatic damage, are thought to be involved (reviewed in Slusarenko *et al.* 1991). Keppler and Novacky (1986) showed that during the hypersensitive response (HR) membrane lipid peroxidation occurred, and increases in lipoxygenase enzyme activity have been reported in several incompatible host-pathogen interactions (see Slusarenko *et al.* 1991, 1993 for reviews). We showed that lipoxygenase activity increased during the HR in bean and postulated that lipoxygenase, perhaps working in conjunction with lipolytic acyl hydrolase, might cause HR membrane damage (Croft *et al.* 1990; Slusarenko *et al.* 1991). The fatty acid hydroperoxide products formed from LOX activity, or their metabolites, have been postulated to produce signal molecules which might coordinate plant defense responses (Farmer and Ryan 1992) and provide substances which can directly inhibit pathogens (Croft *et al.* 1993).

Thus, there are many ways in which LOX might be important in plant-pathogen interactions (reviewed in Slus-

arenko *et al.* 1993). To better understand the LOX response we cloned and characterized a LOX cDNA from bean and used it to investigate the temporal and spatial induction of LOX transcripts in and around bacterially induced hypersensitive lesions.

The precise location of defense gene activation in relation to the site of inoculation of a pathogen and the part played by the rapid, localized host hypersensitive response (HR) in coordinating or amplifying defense responses have long been questions of interest in plant pathology. Biochemical investigations of biosynthetic enzyme capacity and labeled-precursor feeding experiments (Nakajima *et al.* 1975; Rathmell and Bendall 1971; Uritani *et al.* 1976) suggested that although phytoalexins accumulated in necrotic lesions at the site of pathogen invasion, their biosynthesis occurred, at least in part, in the surrounding healthy cells (Bailey 1982). The report that activation of chalcone synthase (CHS) promoters occurred in a zone of host cells outside the area that had been inoculated with pathogens or elicitor preparations is consistent with this idea (Schmid *et al.* 1990; Stermer *et al.* 1990). The phytoalexins that accumulate in bean are isoflavonoids and CHS is a key enzyme in their biosynthesis. To better understand the nature of plant defense and how the various responses are interrelated, several studies have been undertaken to localize the cells in which defense genes are activated. This has been done either by quantifying defense gene transcripts after localized tissue sampling (Bell *et al.* 1984), *in situ* hybridization (Cuypers *et al.* 1988; Schmelzer *et al.* 1989; Schröder *et al.* 1992) or using defense-gene-promoter/reporter gene fusions for histochemical studies (Stermer *et al.* 1990; Roby *et al.* 1990). The majority of these studies involve fungal infections of plants.

When leaf panels are inoculated by syringe with cells of an avirulent or nonhost isolate of a plant pathogenic bacterium at relatively high inoculum concentration, leaf tissue in the inoculated area undergoes a macroscopic hypersensitive reaction (HR), and the discrete area of the inoculated panel collapses and dries out, browning as it does so. Of particular interest is the way in which the hypersensitive reaction (HR) relates to other defense responses like the accumulation of phytoalexins and potentially antimicrobial hydrolases. Thus, although defense gene activation can precede cell necrosis (Templeton and Lamb 1988, Slusarenko *et al.* 1991), as indeed is illustrated in the present work, phytoalexins do not tend to accumulate in whole tissues unless host cell necrosis is apparent (Lyon and Wood 1975; Wyman and Vanetten 1982). Similarly, inoculation of leaves with saprophytic bacteria, which do not elicit an HR (Klement 1963; Klement and Goodman 1967), was shown not to lead to phytoalexin accumulation (Keen *et al.* 1981; Lyon and Wood 1975). On the basis of such observations it was suggested that the HR might serve to release endogenous elicitors (Bailey 1982) which coordinate and perhaps amplify the initial response that occurs in cells directly stimulated by the pathogen to the adjacent host cells (Collinge and Slusarenko 1987; Slusarenko *et al.* 1991). The availability of probes for genes on the biosynthetic pathway leading to phytoalexin accumulation has now enabled not only the phytoalexin end products to be measured, but also the accumulation of mRNA coding for some of the enzymes on the biosynthetic pathway. Thus, it was shown by Jakobek and Lindgren (1993) and Jakobek *et al.* (1993) that transcripts for PAL, CHS, chalcone isomerase (CHI), and CHT accumulated after vacuum-infiltration of bean leaves with cells of a *P. tabaci* Hrp$^-$ mutant and *E. coli*. Interestingly, and in contrast to the reports cited above, these authors also observed the accumulation of antifungal phytoalexins in response to *E. coli*, other saprophytes such as *P. fluorescens*, and the *P. tabaci* Hrp$^-$ mutant.

In the present report we describe the temporal and spatial accumulation of transcripts for defense genes associated with phytoalexin synthesis in bean (PAL and CHS), and in addition, chitinase (CHT) and lipoxygenase (LOX), by localized tissue sampling of bean leaves inoculated with virulent or avirulent isolates of the bacterial bean pathogen, *Pseudomonas syringae* pv. *phaseolicola* or the saprophyte *P. fluorescens*. Our results support some of the recent findings of Jakobek and Lindgren (1991, 1993) and Jakobek *et al.* (1993), but disagree in some aspects. Thus, as reported earlier for *P. vulgaris* (Lyon and Wood 1975) and soybean (Keen *et al.* 1981), we found no accumulation of phytoalexins after inoculation of bean leaves with cells of the saprophyte *P. fluorescens*. The results are discussed in relation to the site and coordination of defense responses in plants.

RESULTS

Microscopy.

Figure 1A shows the sampling zones 1, 2, and 3 in primary leaves inoculated with a hypodermic syringe. Avirulent cells of race 1 of *Pseudomonas syringae* pv. *phaseolicola* induce hypersensitive cell collapse visible from approximately 18 hr postinoculation (hpi) of Red Mexican bean leaves. HR cell collapse is confined to the directly inoculated zone 1 area of the leaf. Trypan blue staining (Keogh *et al.* 1980; Slusarenko and Longland 1986) revealed that by 24 hpi in the incompatible interaction many nonstained cells were interspersed with large numbers of dead, stained cells in zone 1 (Fig. 1B). In the compatible interaction after inoculation with cells of a virulent race 3 isolate of *P. phaseolicola* there is no apparent trypan blue staining at 24 hpi (Fig. 1C). Similarly, leaves inoculated with the saprophyte *P. fluorescens* showed only a few cells which stained with trypan blue (Fig. 1D).

Accumulation of transcripts for PAL.

PAL mRNA had accumulated in the incompatible interaction in zone 1 by 3 hpi and transcript levels peaked at 14—15 hpi (Fig. 2A). Indeed, a weak signal was observable by 1 hpi, which was the earliest time point tested (data not shown). From 3 to 6 hpi transcript levels seemed to decline and begin increasing again, with minor fluctuations up to the maximum. There was some accumulation of PAL transcripts in zone 2, also peaking at approximately 12–15 hpi, but this was minor in comparison to accumulation in zone 1. Indeed, although a weak signal was visible on the autoradiographs, this has not reproduced well on the photographs (Fig. 2A). No signal was seen from zone 3 (Fig. 2A).

In the compatible interaction, PAL transcripts began to accumulate about 15 hpi and increased up to approximately 24 hpi but had begun to decrease by 29 hpi (Fig. 2B). Zones 2 and 3 showed no accumulation of PAL transcripts in the compatible interaction up to 29 hpi.

Transcripts for PAL did not accumulate after buffer treatment of leaves.

Accumulation of transcripts for CHS.

CHS transcripts had begun to accumulate by 3 hpi in zone 1 from the incompatible interaction, and transcript levels showed a broad plateau between 8–18 hpi before declining somewhat (Fig. 2A). Essentially, no accumulation of CHS transcripts was observed in zones 2 or 3 (Fig. 2A). Vacuum-infiltrated leaves showed a somewhat similar pattern (Fig. 3A). No accumulation of CHS transcripts was observed in zones 2 or 3 up to 29 hpi. In the compatible interaction CHS transcripts accumulated very weakly in a transient manner around 18 hpi in zone 1 (Fig. 2B). No accumulation of CHS transcripts was observed in zones 2 or 3 up to 29 hpi.

The accumulation of CHS transcripts also occurred in leaves inoculated with cells of *P. fluorescens*, a saprophyte that caused no macroscopically visible response in the leaves. The accumulation of CHS transcripts was not as great as in the incompatible interaction but was similarly only observed in zone 1 (Fig. 4).

Transcripts for CHS did not accumulate after buffer treatment of leaves.

Accumulation of transcripts for CHT.

CHT transcripts began to accumulate between 3–6 hpi and reached a maximum by approximately 10 hpi in zone 1 of the incompatible interaction, where levels remained high until the end of the experiment (Fig. 3A). Weak, transient accumulation of CHT transcripts was seen in zone 2 between 10–15 hpi (Fig. 2A). Vacuum infiltrated leaves behaved as zone 1 in syringe-inoculated leaves (Fig. 3A).

In the compatible interaction CHT transcripts began to accumulate in zone 1 from 14 hpi onwards (Fig. 2B). No accumulation of CHT transcripts was observed in zones 2 or 3 up to 29 hpi. In vacuum-infiltrated leaves CHT

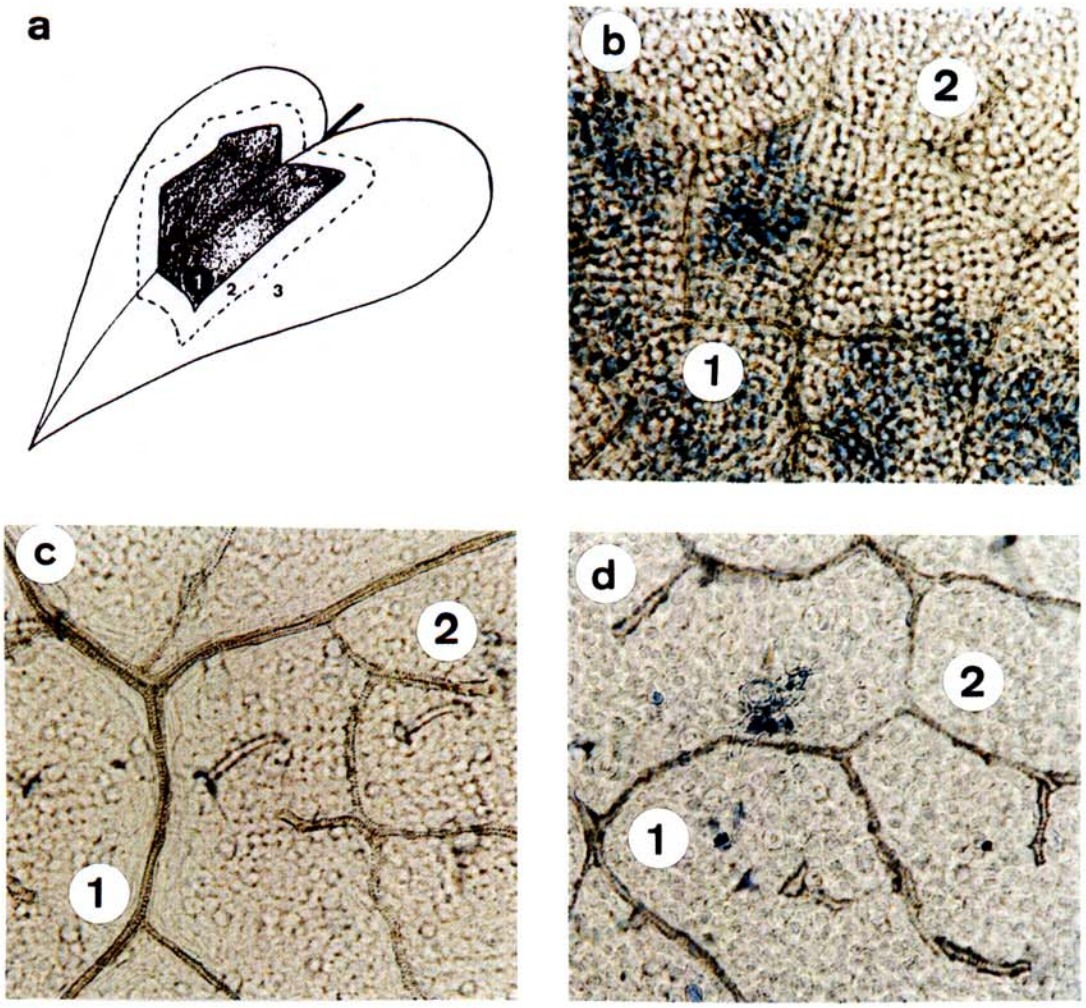

Fig. 1. A, Diagram showing the sampling zones: zone 1 (inoculated area), zone 2 (surrounding 5–7 mm), and zone 3 (remainder of the leaf). Insets show the appearance of cells at the border between zones 2 and 3 after trypan blue staining 24 hr after inoculation with **B,** avirulent bacteria (incompatible interaction), **C,** virulent bacteria (compatible interaction), and **D,** the saprophyte *Pseudomonas fluorescens*.

transcripts were first visible in the compatible interaction from 18 hpi onwards (Fig. 3B).

The accumulation of CHT transcripts also occurred in leaves inoculated with cells of *P. fluorescens* (Fig. 4). As in inoculations with plant pathogenic bacteria, the accumulation of CHT transcripts was only observed to any degree in zone 1.

Transcripts for CHT did not accumulate after buffer treatment of leaves.

Accumulation of transcripts for LOX.

The nucleotide and amino acid sequences of the 2.2-kb partial cDNA clone for bean LOX (pLOX3) used in this work are shown in Figure 5. The presence of the typical LOX iron-binding-site is indicated (Bairoch 1990). The clone has approximately 76% identity at the nucleotide and amino acid levels to other published LOX sequences and a further 10–12% similarity when conservative amino acid substitutions are taken into account (Shibata *et al.* 1987, 1988; Yenofsky *et al.* 1988).

Young primary leaves of bean have a background level of LOX transcripts, which declines as the leaves age (Eiben *et al.* 1993). The background level of LOX transcripts at the beginning of an experiment varied from batch to batch of plants and also to some extent between plants in a single experiment. This complicated the estimation

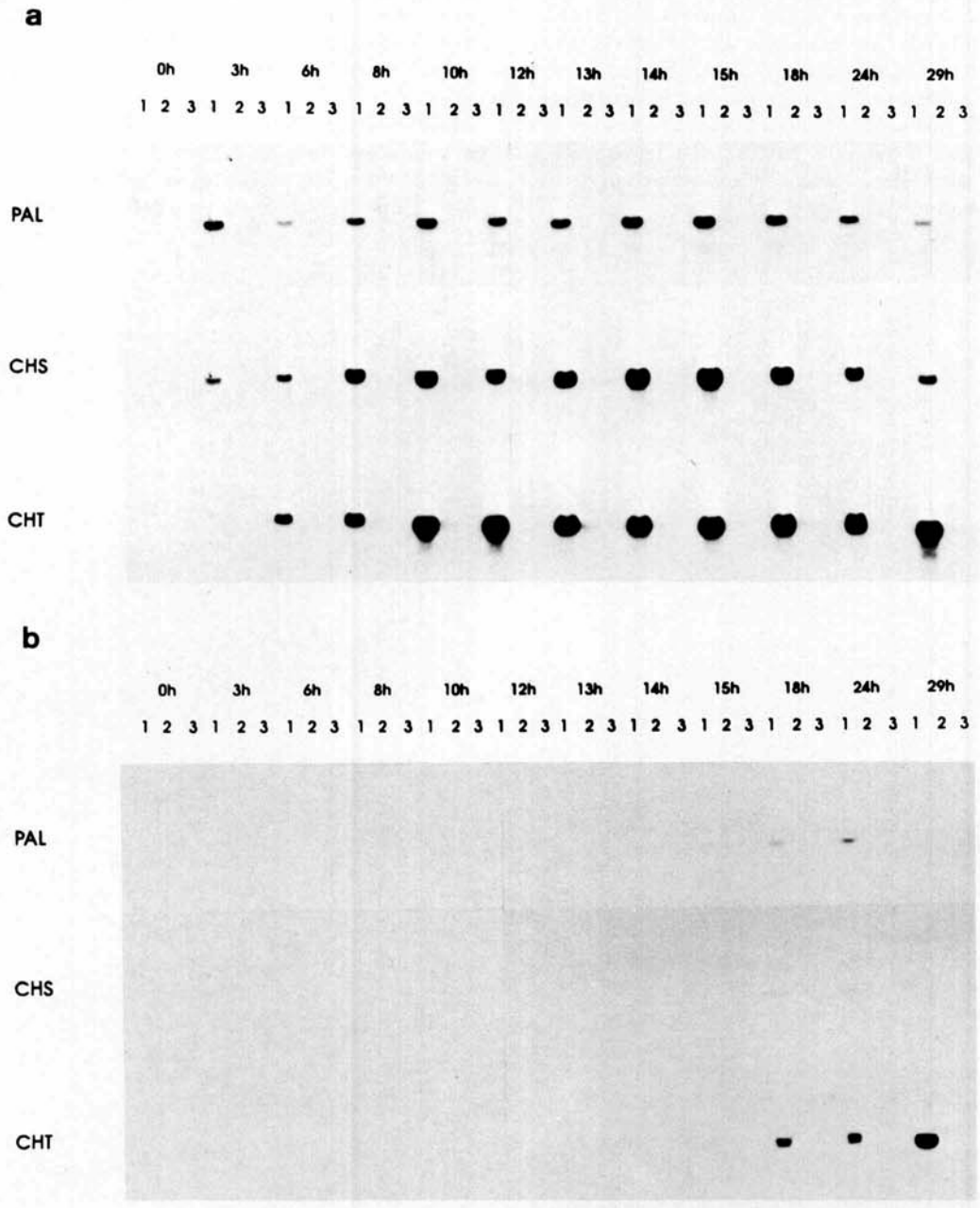

Fig. 2. Northern blot of total RNA isolated at various times (hr) after syringe-inoculation from zones 1, 2, and 3, probed with bean cDNA probes for phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), and chitinase (CHT), respectively. **A,** Incompatible interaction (HR), **B,** compatible interaction (susceptible).

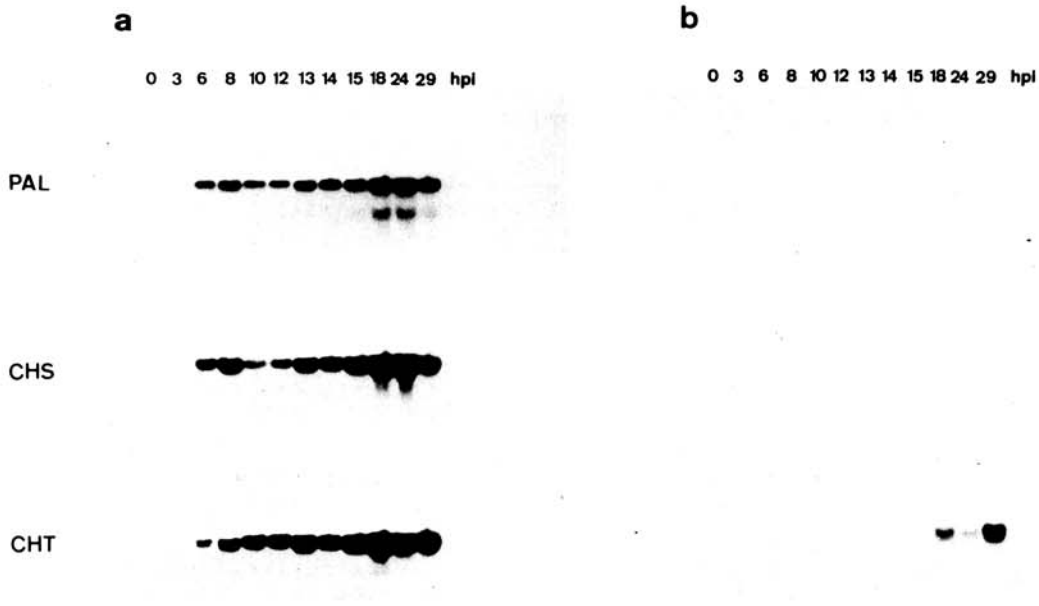

Fig. 3. Northern blot of total RNA isolated at various times (hr) after vacuum infiltration of bacteria into the whole leaf, probed with bean cDNA probes for phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), and chitinase (CHT), respectively. **A,** Incompatible interaction (HR), **B,** compatible interaction (susceptible).

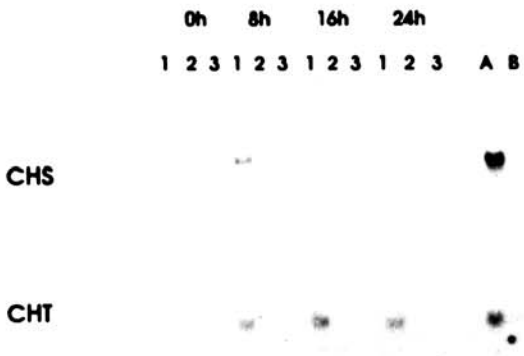

Fig. 4. Northern blot of total RNA from zones 1, 2, and 3 isolated at various times (hr) after syringe inoculation of *Pseudomonas fluorescens* into the leaf, and probed with bean cDNA probes for chalcone synthase (CHS) or chitinase (CHT). Positive controls of RNA from "A" the incompatible interaction (15 hpi), and "B" the compatible interaction (24 hpi) are included for comparison.

of changes in LOX transcript levels in the first 14 hpi, where transcript levels were relatively low compared to the strong induction seen later. However, in spite of these difficulties, some general trends were discernible and were characteristic for either the compatible or incompatible interactions. Thus, the normal decline in LOX transcripts as leaves age was accelerated over the first 12 hpi with avirulent bacteria (Figs. 6, 7). In contrast, a weak, transient induction peaking at 5–6 hpi was observed in the compatible interaction in some inoculations but not in others. In contrast to transcripts for PAL, CHS, and CHT, which accumulated only in zone 1 to any great degree, transcripts for LOX accumulated predominantly in zones 2 and 3. Inoculation with either virulent or avirulent bacteria caused pronounced LOX transcript accumulation in zones 2 and 3 after approximately 14 hr, but avirulent bacteria did not induce accumulation in zone 1, whereas virulent bacteria did (Figs. 6A,B, 7A,B). Buffer treatment also resulted in a jump in transcript levels after approximately 14 hr (Fig. 8), thus indicating that the inoculation procedure itself, or the transient anoxia associated with it, might be sufficiently stressful to cause accumulation of LOX transcripts (Maccarrone *et al.* 1991).

Phytoalexins.

Substances with UV-fluorescence characteristic of isoflavonoid phytoalexins in bean had begun to accumulate to detectable levels only in zone 1 from the incompatible interaction by 24 hpi and amounts continued to increase up to 48 hpi (Fig. 9). In the present work no attempt was made to further identify these substances because we and other workers have done this on numerous previous occasions (Ingham 1982; Slusarenko *et al.* 1989; Longland *et al.* 1992). Presumably, the nonstained, still living cells are the sites of phytoalexin synthesis in zone 1 after HR cell collapse, i.e., accumulation after 18–24 hpi. It seems highly improbable that phytoalexins could be synthesized in the dead cells from remote precursors because these cells can be presumed to be metabolically incompetent. It is quite clear that no phytoalexins accumulated in leaves up to 48 hpi with cells of either a virulent isolate of *P. s.* pv. *phaseolicola* or the saprophyte *P. fluorescens* (Fig. 9).

DISCUSSION

PAL, CHS, CHT, and phytoalexins.

We have previously followed the kinetics of PAL, CHS, and CHT transcript accumulation during the HR in bean leaves vacuum-inoculated with cells of *P. s.* pv. *phaseolicola* (Slusarenko *et al.* 1991; Voisey and Slusarenko 1989).

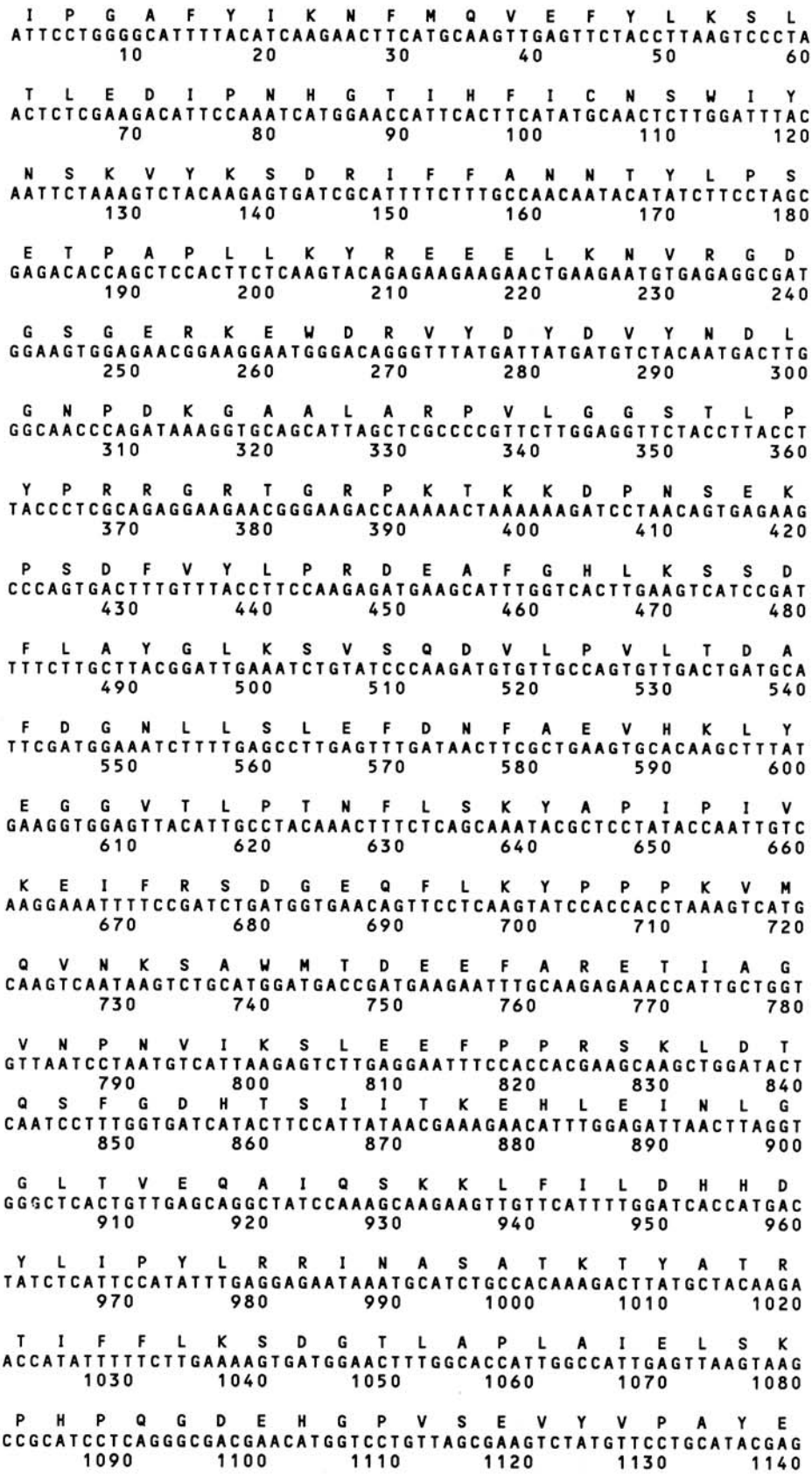

Fig. 5. Nucleotide sequence of a partial cDNA clone of liporygenase from bean. The amino acid sequence is written above the nucleotide sequence. The position of the lipoxygenase iron-binding signature is indicated by a solid horizontal bar above the sequence. (*Continued on next page*)

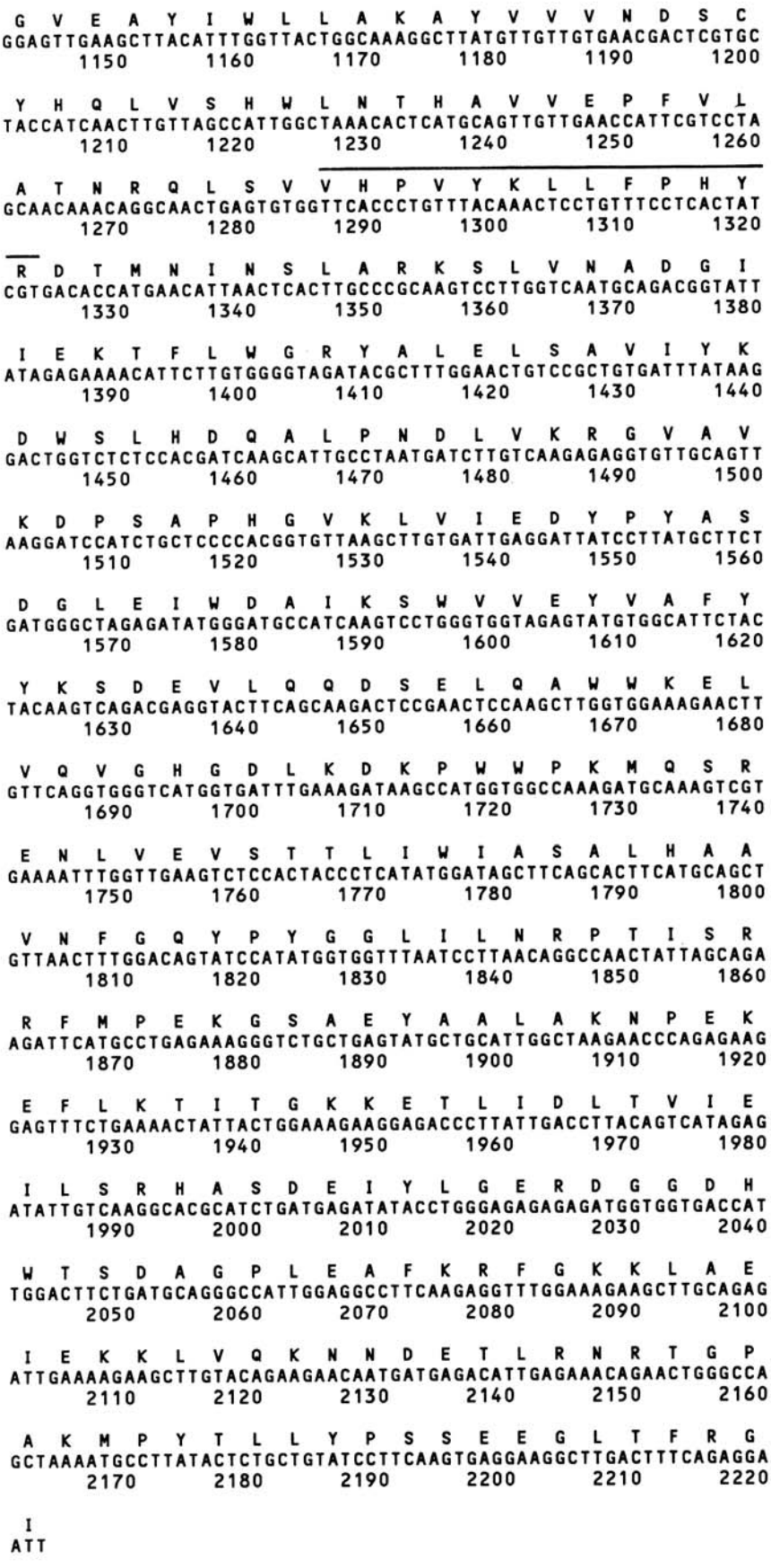

Fig. 5. (*Continued from preceding page*)

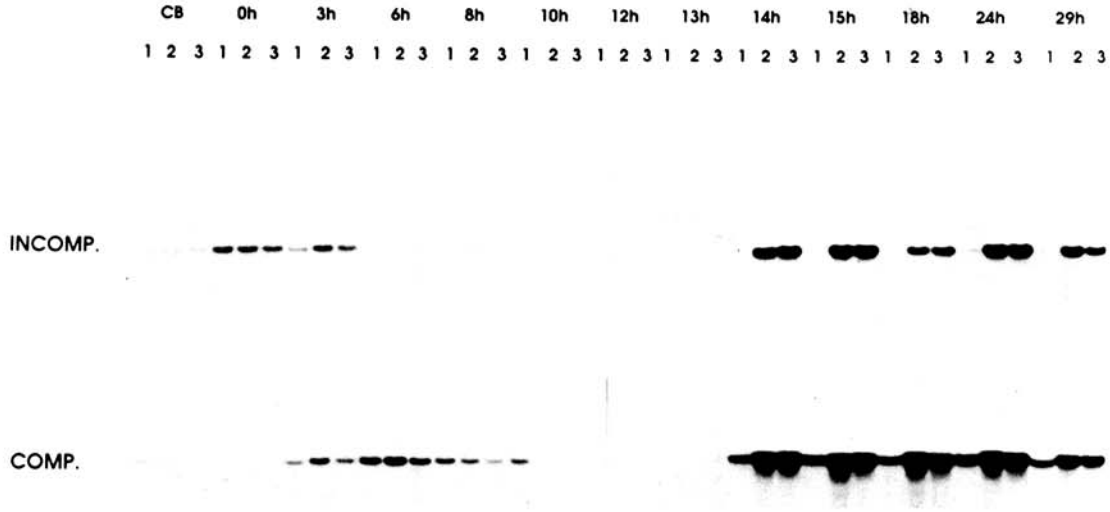

Fig. 6. Northern blot of total RNA isolated from zones 1 (site of inoculation), 2 (surrounding 5–7 mm), and 3 (rest of the leaf) at various times after syringe-inoculation with bacteria, probed with the insert from pLOX3. CB is a buffer-inoculated control. Incomp. = Incompatible interaction (HR), Comp. = compatible interaction (susceptible).

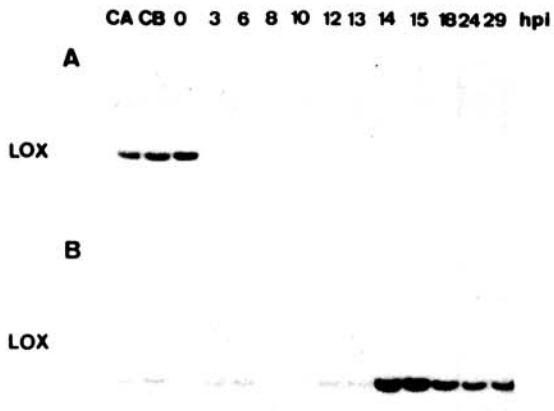

Fig. 7. Northern blot of total RNA isolated at various times after vacuum infiltration of bacteria into the whole leaf, probed with the insert from pLOX3. CA and CB are time 0 noninoculated and buffer-inoculated controls, respectively, and illustrate, in comparison with the time 0 bacteria-inoculated controls, the variation in background LOX signal. **A,** Incompatible interaction (HR), **B,** compatible interaction (susceptible).

After vacuum infiltration with avirulent bacterial isolates, whole leaves show a mosaic of HR-collapsed and healthy tissue which is not suitable for localizing the cells in which defense gene activation occurred. However, the sharply localized nature of the HR after syringe-inoculation with bacteria enabled us to sample discrete, concentric areas of leaf tissue and compare transcript accumulation between them (Fig. 1A). This enabled us to follow the kinetics of the host response to infection in a plant which cannot be efficiently transformed and is thus unsuitable for studies using promoter-reporter gene fusions.

Our working hypothesis was that we could expect to observe accumulation of (at least) PAL and CHS transcripts in plant cells in zone 2 where phytoalexin synthesis

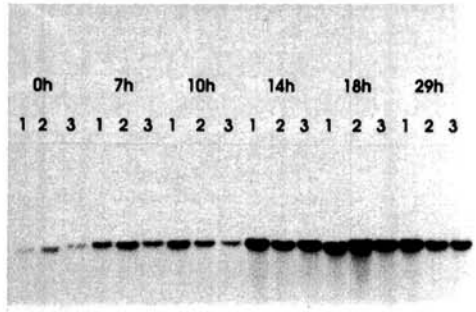

Fig. 8. Northern blot of total RNA isolated from zones 1 (site of inoculation), 2 (surrounding 5–7 mm) and 3 (rest of the leaf) at various times after syringe-infiltration with buffer, probed with the insert from pLOX3.

was assumed to occur adjacent to the HR lesion (see introduction). Thus, it was somewhat surprising to us that up to 29 hpi, with HR occurring at approximately 18–20 hpi, we detected accumulation of PAL and CHS transcripts almost exclusively in the directly inoculated zone 1 tissues and not in the surrounding tissues (Fig. 2A). In comparison there was only a very slight accumulation of PAL and CHS transcripts in zone 2, which bordered on the inoculated zone. Bell *et al.* (1984) presented similar results showing accumulation of CHS transcripts at hypersensitive lesions in bean hypocotyls inoculated with conidial suspensions of *Colletotrichum lindemuthianum*. However, in their case it was not possible to excise the HR lesion precisely, and their zone 1 samples were standardized to include a block of adjacent healthy tissue (Lamb *et al.* 1986).

Stermer *et al.* (1990) investigated the activation of the bean CHS8 and CHS15 promoters in response to various stimuli in transgenic tobacco. The promoters were fused to the *E. coli uidA* (GUS) reporter and interestingly, although the CHS15 promoter was strongly induced in bean by elicitor treatment (Ryder *et al.* 1987), in tobacco there was no induction by elicitor or inoculation with the nonhost pathogen *P. s.* pv. *syringae*. There was weak

induction of the bean CHS8 gene promoter in the transgenic tobacco in tissue surrounding inoculation sites of *P. s.* pv. *syringae*. However, it is difficult to compare the results of Stermer *et al.* directly with our results. Firstly, they studied the regulation of the genes in a nonhomologous system, i.e., a bean promoter in tobacco, and secondly, only two CHS promoters were examined of the seven members of the bean CHS gene family. Thus, it is possible that other bean CHS promoters would have behaved differently in response to elicitor and pathogen treatment. However, in principle, the results of Stermer *et al.* (1990) are in agreement with our observation that very little CHS gene activation occurs in zones 2 and 3 (Fig. 2A). Stermer *et al.* did not measure *uidA* transcripts, and after cell collapse it is not possible to demonstrate GUS activity in the lesion itself because the integrity of the plant tissue within the lesion is lost during the incubation with the substrate. Thus, it is not possible to say if the induction of CHS8 promoter in the lesion was comparable to that which would be expected from the accumulation of transcripts we observed in zone 1 in inoculated bean leaves. On the other hand, studies with plant pathogenic fungi have shown pronounced accumulation of PAL transcripts in the cells adjacent to inoculation sites (Cuypers *et al.* 1988; Schmelzer *et al.* 1989; Schröder *et al.* 1992). Thus, it could be argued that in our work, contamination of zone 1 tissue with cells on the border of zone 2 might be the source of the defense-related transcripts in our zone 1 samples. However, this possibility can be ruled out because when zone 1 tissue was excised well within the necrotic border of zone 1, the signals obtained from the defense transcripts was unaltered (data not shown). Light microscopy after trypan blue staining showed that at 24 hpi in the HR lesion (zone 1), living (nonstaining) cells were interspersed among the dead, stained cells (Fig. 1B).

Phytoalexins are first detectable in bean in low concentrations at 24 hpi in the incompatible interaction and reach a maximum by 48 hpi (Fig. 9 and Lyon and Wood 1975). Thus, we are faced with the apparent paradox that at the major site of defense gene activation, the majority of cells die before phytoalexin synthesis has really started, and the surrounding cells in zone 2 do not seem to take part—at least at the level of transcript accumulation. It is possible that phytoalexins are synthesized only in the remaining living cells interspersed among the dead cells in zone 1. It seems inconceivable that the synthesis of phytoalexins from their remote precursors could occur in cells that have already undergone HR cell collapse. The surrounding healthy tissue might to some extent use preexisting enzymes to synthesize phytoalexins rather than relying on *de novo* enzyme synthesis. This would be in agreement with the results of the labeled precursor feeding experiments mentioned in the introduction (Nakajima *et al.* 1975; Rathmell and Bendall 1971; Uritani *et al.* 1976).

It is clear that the major accumulation of PAL, CHS, and CHT transcripts occurs in zone 1. Interestingly, the inverse was found when the same blots were probed with a bean lipoxygenase cDNA. This demonstrates that the observed spatial pattern of transcript accumulation is real and not a treatment or sampling artifact. In general, vacuum-infiltrated whole primary leaves behaved like the directly inoculated tissue in zone 1 of syringe-inoculated leaves (compare Figs. 2 and 3).

Studies with phytopathogenic fungi showed that in compatible interactions chitinase promoters were activated in cells in the zone adjacent to infection sites (Roby *et al.* 1990; Samac and Shah 1991). Whether there is a generally wider, local systemic induction of defense genes such as PAL, CHS, and CHT in fungus-plant interactions compared to bacteria-plant interactions, remains to be tested. With respect to the differential accumulation of CHT transcripts in the *P. vulgaris*/*P. s.* pv. *phaseolicola* pathosystem, the present results confirm and extend those reported earlier for vacuum-infiltrated primary bean leaves (Voisey and Slusarenko 1989). Chitinase transcripts began to accumulate well before HR cell collapse in zone 1 in the incompatible interaction. Thus, our results also lend support to the idea that, at least in bean, it is the release of a concentrated burst of accumulated vacuolar chitinase upon HR cell collapse that might be important for plant

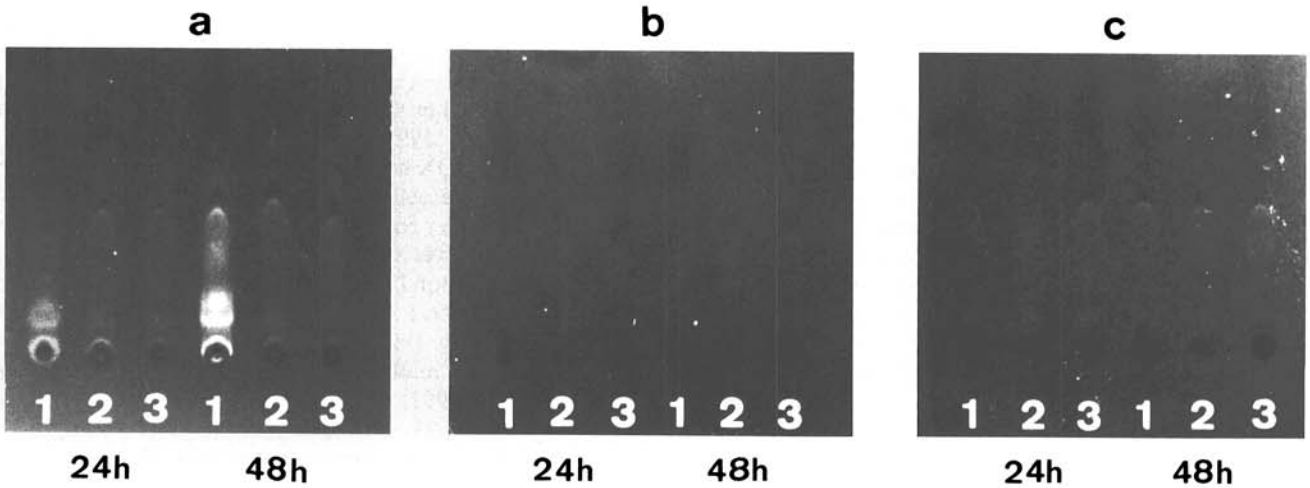

Fig. 9. Thin-layer chromatographs of UV-fluorescing substances from zones 1, 2, and 3, at 24 and 48 hr after inoculation with either **A,** avirulent, **B,** virulent cells of *Pseudomonas syringae* pv. *phaseolicola* or **C,** saprophytic (*Pseudomonas fluorescens*).

defense and not a gradual accumulation of extracellular chitinase activity (Boller 1987, 1993).

It was reported recently (Jakobek and Lindgren 1991, 1993; Jakobek *et al.* 1993) that saprophytic *P. fluorescens*, heat killed cells of *P. s.* pv. *phaseolicola*, a *P. tabaci* Hrp$^-$ mutant and *E. coli*, all of which do not elicit an HR, induced the accumulation of PAL and CHS transcripts and caused accumulation of phytoalexins. These reports have bearing on interpreting the relationship the HR has to other defense responses. Although activation of some defense genes occurs before HR cell death (Templeton and Lamb 1988), phytoalexins were usually not found to accumulate in whole plant tissues in the absence of host cell necrosis (Keen *et al.* 1981; Lyon and Wood 1975; Lyon and Wood 1976; Wyman and Vanetten 1982). In this work we also observed an accumulation of CHS and CHT transcripts in zone 1 after inoculation with *P. fluorescens*, albeit weaker than in the incompatible interaction, but we detected no accumulation of antibacterial isoflavonoid phytoalexins (Figs. 4, 9). Accumulation of UV fluorescent substances, matching those previously shown to have phytoalexin activity (Longland *et al.* 1987; Longland *et al.* 1992; Lyon and Wood 1975; Slusarenko *et al.* 1989), were only detected in the 24 and 48 hpi zone 1 samples from the incompatible interaction. This suggests that either the accumulation of phytoalexin-related transcripts induced by *P. fluorescens* is quantitatively insignificant, or that there is a later control step, which is necessary for phytoalexin accumulation. This would be analogous to the early and late groups of coordinately regulated genes involved in anthocyanin biosynthesis in *Antirrhinum majus* (Jackson *et al.* 1992). Here it was shown that PAL, CHS, and CHI (chalcone isomerase) were coordinately induced early on, whereas flavonone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), and UDP glucose:flavonoid 3-*O*-glucosyl transferase (UFGT) were coordinately induced later on. The authors suggest that the simplest model to explain coordinate regulation of genes is that they all respond to the same signals using the same regulatory factors and that different groups are governed by different temporal control mechanisms. In our case, the presence of avirulent or saprophytic bacteria in the leaves obviously provides a signal for the induction of some defense gene transcripts involved in early steps of phenylpropanoid metabolism (PAL and CHS), but some genes involved in the later production of the isoflavonoid phytoalexins are perhaps not induced, or the enzymes are regulated in some other manner such that the metabolic flux through to phytoalexins does not occur.

Since only scattered single cell death was apparent in trypan blue-stained tissue after inoculation with *P. fluorescens* (Fig. 1D), this postulated later control step seems to correlate with the macroscopic necrosis occurring as a result of the HR and may depend on signals produced by dying cells (Slusarenko *et al.* 1991; Koch *et al.* 1992). Investigation of the regulation of genes involved in the later stages of isoflavonoid biosynthesis after inoculation with pathogenic and saprophytic bacteria will shed further light on this hypothesis. A similar suggestion that enzymes later than PAL and CHS in the biosynthetic pathway might play a key regulatory role in glyceollin synthesis in soybean, has already been made (Smith and Banks 1986). Why the results in this and other studies (Keen *et al.* 1981; Lyon and Wood 1975) differ from those of Jakobek and Lindgren (1991, 1993) with respect to phytoalexin accumulation is not clear. It is possible that some unknown stimulus, perhaps due to different experimental procedures, activated the later steps necessary for isoflavonoid biosynthesis. The vacuum-infiltration procedure is not likely to be the cause because no phytoalexins accumulated in bean leaves after vacuum infiltration with UV- or streptomycin-killed *P. s.* pv. *phaseolicola* (data not shown). One possibility is the effect of sunlight on the plants, which were greenhouse-grown until transferring to a growth cabinet shortly before inoculation (Jakobek *et al.* 1993). Certainly, light (UV and blue light) can have pronounced effects on plant phenolic metabolism in general and will induce the synthesis of anthocyanins (Wellmann *et al.* 1976). However, our data do support the conclusions of Jakobek and Lindgren (1991, 1993) that saprophytes can activate at least some defense genes in bean.

Lipoxygenase.

If the hypothesis that LOX activity is important in causing membrane damage in the HR and in producing signal molecules for the coordination of plant defense responses is correct (Croft *et al.* 1990; Koch *et al.* 1992; Slusarenko *et al.* 1993), one might expect LOX transcripts to increase in those cells destined to undergo HR cell collapse but not in surrounding healthy cells and in host cells in contact with virulent bacteria (Slusarenko *et al.* 1991). Essentially the opposite of this was observed in the spatial and temporal accumulation of LOX transcripts in bean leaves inoculated with an avirulent race of *P. s.* pv. *phaseolicola* (Figs. 6A,B, 7A,B). Indeed, the pattern is remarkable because the inverse distribution was observed for phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), and chitinase (CHT) transcripts. Thus, although there appear to be pathosystem-specific changes in LOX transcript accumulation in bean, the patterns seen were unexpected. The pattern of LOX transcript accumulation observed in the HR has features in common with that which might be expected for a vegetative storage protein (VSP) form of the enzyme (Tranbarger *et al.* 1991). Thus, transcripts are abundant in young leaves, the amount decreases as the leaves mature (Eiben *et al.* 1993), and VSP accumulation is induced by such stresses as wounding and drought (Staswick *et al.* 1991). In response to pathogen stress a VSP form of LOX might be induced to accumulate except in tissues where cells are fully switched over to defense metabolism, i. e., zone 1 in the incompatible interaction.

In tomato leaves vacuum infiltrated with pseudomonad cells, induction of LOX activity, mRNA and protein all changed in concert and correlated well with HR cell collapse (Koch *et al.* 1992). Similar coordinated changes were shown for LOX in other pathosystems, e.g., rice/rice blast (Shibata *et al.* 1991, 1993) and *Arabidopsis*/*Pseudomonas* (Melan *et al.* 1993). The latter hosts are all nonlegumes, and it may be that the complex nature of LOX gene families in legumes makes these plants difficult subjects for studies on LOX regulation in response to pathogens and other stimuli. Several LOX isozymes are highly homologous at

the amino acid and nucleotide levels. Indeed, the majority of LOX's so far described show 60–70% or greater identity with each other. The bean cDNA we isolated falls well within this group, showing 76% identity at the nucleotide level with soybean LOX-3 (Yenofsky *et al.* 1988). Ohta *et al.* (1992) showed the activation of a lipoxygenase pathway in rice after infection with *Magnaporthe grisea* and recently Shibata *et al.* (1993) showed that the gene responsible for this activity encodes a form with low amino acid identity with other LOX sequences. Biochemical studies with bean showed clearly that the membrane fatty acid peroxidation that accompanies HR cell collapse is mediated by lipoxygenase activity, is under tight metabolic control, and is not the result of autoxidation (Croft *et al.* 1993). This suggests that the lipoxygenase important in the HR in bean remains to be identified and may, as in rice, fall into a class with less sequence homology to the LOX's so far described.

Further work to purify the specific LOX enzyme(s) important in the HR, and employing gene-specific probes, is in progress, and it is hoped will eventually lead to a better understanding of the role of LOX in plant defense.

MATERIALS AND METHODS

Plant material.

P. vulgaris (L.) 'Red Mexican U.I.3' seedlings were grown in a growth chamber with a 16 hr light and 8 hr dark period and day and night temperatures of 25 and 23° C, respectively, and a light intensity at leaf level of 150 μE $m^{-1}s^{-1}$. Ten-day-old seedlings were inoculated either by vacuum infiltration as described previously (Slusarenko and Longland 1986) or by hypodermic syringe fitted with a gauge 30 needle. Inoculated plants were returned to a growth chamber with constant lighting for the period of the experiment.

Bacteria.

P. s. pv. *phaseolicola* isolates 31A (race 1) and 1301A (race 3) were kindly supplied by J. D. Taylor, IHR, Wellesbourne, U.K., the *P. fluorescens* isolate used in this work originated from the culture collection of the Dept. of Pure and Applied Biology, Imperial College, University of London, U.K. Inoculation of cv. Red Mexican with avirulent race 1 isolates (incompatible interaction) leads to a hypersensitive reaction, whereas virulent race 3 isolates produce spreading, water-soaked lesions.

Working stocks were grown on nutrient agar (Oxoid) supplemented with 1% (v/v) glycerol at 25° C overnight and stored at 4° C. Long-term storage was as glycerol stocks at −80° C. Inocula were grown for approximately 18 hr in King's B medium (King *et al.* 1954) in an orbital shaker at 250 rpm at 25° C. The cultures were centrifuged at 3,000 *g* for 10 min and the bacterial pellet was resuspended gently in inoculation buffer (1.4 mM KH_2PO_4, 2.5 mM Na_2HPO_4, pH 7.0). The inoculum was adjusted to OD_{600} 0.06, which dilution plate counts showed to be approximately 1×10^8 cfu cm^{-3}.

cDNA cloning and library screening.

LiCl-precipitated total RNA from bean leaves, 6 hpi with avirulent bacteria, was subjected to two rounds of oligo(dT) chromatography and poly(A)$^+$ RNA denatured for 5 min at 65° C and cooled rapidly on ice. First-strand cDNA synthesis was carried out with AMV reverse transcriptase (Gibco-BRL, Paisley, U.K.) using standard procedures (Huynh *et al.* 1985). Second-strand synthesis with DNA polymerase I was primed with RNA fragments generated by RNAase H according to standard procedures (Gubler 1987). The double-stranded cDNA was blunt ended with T4 DNA polymerase then treated with *Eco*RI methylase and ligated to *Eco*RI linkers (Pharmacia, U.K.) and subsequently digested with *Eco*RI according to standard methods (Wu *et al.* 1987). Excess linkers were then removed by running the mixture over a Biogel A-50m column (Huynh *et al.* 1985). Size-fractionated cDNA was then ligated with *Eco*RI-digested NM 1149 vector, packaged, and plated out with *E. coli* NM514 (*hflA*) cells and an amplified library was used in further work. The library was plated out with *E. coli* K802 cells and plaques were lifted onto Hybond N nylon filters (Amersham, U.K.), and probed with random-primed [^{32}P]dCTP-labeled insert from pCD45, a partial cDNA encoding a liporygenase from pea, kindly supplied by Claire Domoney (John Innes Institute, U.K.) Positive clones were taken through three rounds of plaque purification. DNA was isolated from phage diffusates from confluently lysed plates of NZY medium solidified with 1.5% agarose. Phage were allowed to diffuse into 5 ml of SM buffer per plate for 5 hr at 4° C and 1 ml centrifuged at top speed in the microfuge. The supernatant (0.7 ml) was transferred to a new tube and 0.6 ml of DE 52 (Whatman, equilibrated three times with NZY medium until the pH = 7.0) was added and mixed well. After microfuging at top speed for 5 min, 0.6 ml of the supernatant was mixed with 120 μl of 2.5% (w/v) SDS in 10 mM Tris, 25 mM Na_2EDTA and incubated for 15 min at 65° C. After cooling, 150 μl of 8 M ammonium acetate was added and the mix incubated on ice for 15 min. The mix was microfuged at top speed for 1 min and 0.8 ml of the supernatant mixed with 0.48 ml of isopropanol, left at room temperature for 2 min, then microfuged at top speed for 10 min. After washing with 80% ethanol, the pellet was dissolved in 10 mM Tris-HCl, 1 mM Na_2EDTA pH 8.0. The phage DNA was examined by agarose gel electrophoresis after *Eco*RI digestion, and the largest insert (approximately 2.2 kb) was purified from the gel with GeneClean (BIO 101 Inc., La Jolla, CA) and subcloned into the *Eco*RI site of pBluescript II SK+ to produce the plasmid pLOX3.

Sequencing.

The approximately 2.2-kb insert from pLOX3 was sequenced by the dideoxy method using a Sequenase kit (United States Biochemical Corp., Cleveland, OH). First-strand sequencing utilized the SK primer and sequential exonuclease III deletions and ssDNA rescued from the phagemid by helper phage infection. Sequencing of the second-strand employed dsDNA and synthetic oligonucleotides made with a 391 DNA synthesizer, PCR-Mate (Applied Biosystems, Foster City, CA). The sequence has the EMBL accession no. X63521.

Probes.

The cDNA clones encoding phenylalanine ammonia lyase (pPAL5), chalcone synthase (pCHS1), and chitinase (pCHT12.3) were kindly supplied by C. J. Lamb (Salk Institute, La Jolla, CA). The cDNA for lipoxygenase was cloned as described above. Inserts used for labeling were purified from agarose-gel separations of suitable restriction digests by using GeneClean (BIO 101 Inc, La Jolla, CA). Probes were labeled with [^{32}P]dCTP (New England Nuclear, Regensdorf, Switzerland) using a random primed labeling kit (Boehringer, Mannheim, Germany) as described by the manufacturer.

Isolation of RNA.

Leaf tissue was frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. Frozen tissue (0.5 g) was added to a 1:1 mixture of phenol and 2× NETS (200 mM NaCl, 2 mM Na_2EDTA, 20 mM Tris-HCl pH 7.5,1% [w/v] SDS) preheated to 80° C. The slurry was vortexed briefly, cooled on ice, and centrifuged. The extraction was repeated and nucleic acids precipitated from the aqueous phase with two volumes of ethanol and collected by centrifugation. The nucleic acid pellet was redissolved in water, and the RNA was precipitated by adding an equal volume of 5 M LiCl and incubating on ice overnight. The RNA was collected by centrifugation and redissolved in water. Sodium acetate (pH 5) was added to 0.2 M and the RNA again precipitated with 2 volumes of ethanol. The RNA was redissolved in water, quantified by UV spectrophotometry, and used for gel blot analysis. For Northern blots 10 μg of total RNA was electrophoresed in a 1% (w/v) agarose gel containing 8% (w/v) formaldehyde. Great care was taken to ensure that equal amounts of RNA were loaded on each track; this was checked by ethidium bromide staining, which showed that equal amounts of ribosomal RNA were present. In addition some filters were probed with pH1, a clone of a "constitutive" mRNA kindly provided by C. J. Lamb (Lamb *et al.* 1986). The band highlighted by pH1 is somewhat diffuse on Northern blots, which makes this probe less satisfactory as an internal standard than on dot blots. However, the quantity of this diffuse band was essentially constant in each track (data not shown). After electrophoresis the gel was capilliary-blotted onto nylon filters (Hybond-N, Amersham, U.K.) according to the manufacturer's instructions. Filters were prehybridized for 3 hr at 60° C in 1 M NaCl containing 10% (w/v) dextran sulphate and 0.8% (w/v) SDS. Hybridization was carried out overnight and conditions were the same as for the prehybridization, except that the mixture contained 150 μg ml^{-1} denatured salmon sperm DNA and denatured, labeled probe. In most experiments and unless otherwise stated, probes were labeled by priming with random oligonucleotides using a kit from Boehringer Mannheim. In some experiments probes were labeled by incorporating digoxigenin-dUTP and visualized by antibody-conjugate/chemiluminescent substrate treatment (3-[2′-spiroadamantane]-4- methoxy-4-[3″phosphoryloxy]-phenyl-1,2-dioxetane, AMPPD). After hybridization filters were washed with 2× SSPE containing 0.1% (w/v) SDS for 10 min at room temperature, followed by 1× SSPE, 0.1% (w/v) SDS for 15 min at 60° C and finally with 0.1× SSPE, 0.1% (w/v) SDS for 15 min at 60° C. Autoradiography was performed with Fuji medical X-ray film and intensifier screens at −70° C. All the experiments were repeated at least once and for internal consistency for each given probe, autoradiographs are shown for filters hybridized at the same time with the same probe and exposed to film for the same period. The filters were stripped by placing in boiling 0.1% SDS and cooling to room temperature before being used for reprobing.

Phytoalexin extraction.

Phytoalexins were extracted from 30-mg aliquots of leaf tissue after lyophilization. Extraction into methanol, concentration and TLC in chloroform/methanol (33:1) was essentially as previously described (Longland *et al.* 1987). Plates were viewed under long wave UV light and photographed using 160 Ektachrome film (Kodak) and a 44 filter (Kodak).

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LITERATURE CITED

Bailey, J. A. 1982. Physiological and biochemical events associated with the expression of resistance to disease. Pages 39-65 in: Active Defense Mechanisms in Plants, NATO ASI series, R. K. S. Wood, ed. Plenum Press, New York.

Bairoch, A. 1990. PROSITE: A dictionary of protein sites and patterns, 5th release. University of Geneva.

Bell, J. N., Dixon, R. A., Bailey, J. A., Rowell, P. M., and Lamb, C. J. 1984. Differential induction of chalcone synthase mRNA activity at the onset of phytoalexin accumulation in compatible and incompatible plant-pathogen interactions. Proc. Natl. Acad. Sci. USA 81:3384-3388.

Boller, T. 1987. Hydrolytic enzymes in plant disease resistance. Pages 247-262 in: Plant-Microbe Interactions: Molecular and Genetic Perspectives, Volume 2. T. Kosuge and E. W. Nester, eds. Macmillan, New York.

Boller, T. 1993. Antimicrobial functions of the plant hydrolases, chitinase and β-1,3-glucanase. Pages 391-400 in: Mechanisms of Plant Defense Responses. B. Fritig and M. Legrand, eds. Kluwer Academic Publishers, Dordrecht.

Collinge, D. B., and Slusarenko, A. J. 1987. Plant gene expression in response to pathogens. Plant Mol. Biol. 9:389-410.

Croft, K. P. C., Voisey, C. R., and Slusarenko, A. J. 1990. Mechanism of hypersensitive cell collapse: Correlation of increased lipoxygenase activity with membrane damage in leaves of *Phaseolus vulgaris* (L.) cv. Red Mexican inoculated with avirulent race 1 cells of *Pseudomonas syringae* pv. *phaseolicola*. Physiol. Mol. Plant Pathol. 36:49-62.

Croft, K. P. C., Jüttner, F., and Slusarenko A. J. 1993. Volatile products of the lipoxygenase pathway evolved from *Phaseolus vulgaris* (L.) leaves inoculated with *Pseudomonas syringae* pv. *phaseolicola*. Plant Physiol. 101:13-24.

Cuypers, B., Schmelzer, E., and Hahlbrock, K. 1988. *In situ* localization of rapidly accumulated phenylalanine ammonia-lyase mRNA around penetration sites of *Phytophthora infestans* in potato leaves. Mol. Plant-Microbe Interact. 1:157-160.

Dixon, R. A. 1986. The phytoalexin response: Elicitation, signalling and control of host gene expression. Biol. Rev. 61:239-291.

Dixon, R. A., and Lamb, C. J. 1990. Molecular communication in

interactions between plants and microbial pathogens. Annu. Rev. Plant Physiol. Mol. Biol. 41:339-367.
Eiben, H. G., Sautter, C., and Slusarenko, A. J. 1993. Lipoxygenase in *Phaseolas vulgaris* development. Page 349 in: Mechanisms of Plant Defense Responses. B. Fritig and M. Legrand, eds. Kluwer Academic Publishers, Dordrecht.
Farmer, E. E., and Ryan, C. A. 1992 Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. Plant Cell 4:129-134.
Friend, J. 1976. Lignification in infected tissue. Pages 291-303 in: Biochemical Aspects of Plant-Parasite Relationships. J. Friend and D. R. Threlfall, eds. Academic Press, New York.
Gubler, U. 1987. Second-strand cDNA synthesis: mRNA fragments as primers. Methods Enzymol. 152:330-335.
Huynh, T. V., Young, R. A., and Davis, R. W. 1985. Constructing and screening cDNA libraries in gt10 and gt11 Pages 49-78 in: DNA Cloning Volume 1. D. M. Glover, ed. The Practical Approach Series, IRL Press, Oxford.
Ingham, I. L. 1982. Phytoalexins from the leguminosae. Pages 21-80 in: Phytoalexins. J. A. Bailey and J. W. Mansfield, eds. Blackie, Glasgow.
Jackson, D., Roberts, K., and Martin, C. 1992. Temporal and spatial control of expression of anthocyanin biosynthetic genes in developing flowers of *Antirrhinum majus*. Plant J. 2:425-434.
Jakobek, J., and Lindgren, P. B. 1991. hrp mutants of *Pseudomonas syringae* pv. *tabaci* induce defense responses. Third International Congress of Plant Molecular Biology, (Abstracts), Tucson, Arizona, abstract No. 1249.
Jakobek, J., and Lindgren, P. B. 1993 Generalized induction of defense responses in bean is not correlated with the induction of the hypersensitive reaction. Plant Cell 5:49-56.
Jakobek, J. L., Smith, J. A., and Lindgren, P. B. 1993 Suppression of bean defense responses by *Pseudomonas sryingae*. Plant Cell 5:57-63.
Kauss, H. 1987. Some aspects of calcium-dependent regulation in plant metabolism. Annu. Rev. Plant Physiol. 38:47-92.
Keen, N. T., Ersek, T., Long, M., Bruegger, R., and Holliday, M. 1981. Inhibition of the hypersensitive reaction of soybean leaves to incompatible *Pseudomonas* spp. by blasticidin S, streptomycin or elevated temperature. Physiol. Plant Pathol. 18:325-337.
Keogh, R. C., Deverall, B. J., and Mcleod, S. 1980. Comparison of histological and physiological responses to *Phakopsora pachyrhizi* in resistant and susceptible soybean. Trans. Br. Mycol. Soc. 74:329-333.
Keppler, L. D., and Novacky, A. 1986. Involvement of lipid peroxidation in the development of a bacterially induced hypersensitive response. Phytopathology 76:104-108.
King, E. O., Ward, M. K., and Raney, D. R. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. 44:301-307.
Klement, Z. 1963. Rapid detection of the pathogenicity of phytopathogenic pseudomonads. Nature 199:299-300.
Klement, Z. 1982. Hypersensitivity. Pages 149-177 in: Phytopathogenic Prokaryotes, Volume 2. M. S. Mount and G. H. Lacy, eds. Academic Press, New York.
Klement, Z., and Goodman, R. N. 1967. The hypersensitive reaction to infection by bacterial plant pathogens. Annu. Rev. Phytopathol. 5:17-44.
Koch, E., Meier, B. M., Eiben, H. G., and Slusarenko, A. J. 1992. A lipoxygenase from leaves of tomato (*Lycopersicon esculentum* Mill.) is induced in response to plant pathogenic pseudomonads. Plant Physiol. 99:571-576.
Lamb, C. I., Corbin, D. R., Lawton, M. A., Sauer, N., and Wingate, W. P. M. 1986. Recognition and response in plant:pathogen interactions. Pages 333-344 in: Recognition in Microbe-Plant Symbiotic and Pathogenic Interactions. B. Lugtenberg, ed. Springer-Verlag, Berlin.
Longland, A. C., Slusarenko, A. J., and Friend, J. 1987. Arachidonic and linoleic acids elicit isoflavonoid phytoalexin accumulation in *Phaseolas vulgaris* (French bean). J. Phytopathol. 120:289-297.
Longland, A. C., Slusarenko, A. J., and Friend, J. 1992. Pectolytic enzymes from interactions between *Pseudomonas syringae* pv. *phaseolicola* and French bean (*Phaseolus vulgaris*). J. Phytopathol. 134:75-86.
Lyon, F. M., and Wood, R. K. S. 1975. Production of phaseollin, coumestrol and related compounds in bean leaves inoculated with *Pseudomonas* spp. Physiol. Plant Pathol. 6:117-124.
Lyon, F., and Wood, R. K. S. 1976. The hypersensitive reaction and other response of bean leaves to bacteria. Ann. Bot. 40:479-491.
Lyon, F. M., and Wood, R. K. S. 1977. Alteration of response of bean leaves to compatible and incompatible bacteria. Ann. Bot. 41:359-367.
Maccarrone, M., Veldink, G. A., and Vliegenthart, I. F. G. 1991. Phytochrome control and anoxia effect on the activity and expression of soybean seedling lipoxygenase-1 and lipoxygenase-2. FEBS Lett. 291:117-121.
Melan, M., Dong, X., Endara, M., Davis, K. R., Ausubel, F. M., and Peterman, T. K. 1992. An *Arabidopsis thaliana* lipoxygenase gene can be induced by pathogens, abscisic acid, and methyl jasmonate. Plant Physiol. 101:441-450.
Nakajima, T., Tomiyama, K., and Kinukawa, M. 1975. Distribution of rishitin and lubimin in potato tuber tissue infected by an incompatible race of *Phytophthora infestans* and the site where rishitin is synthesized. Ann. Phytopathol. Soc. Jpn. 41:49-55.
Ohta, H., Shida, K., Peng, Y-L., Furusawa, I., Shishiyama, J., Albara, S., and Morita, Y. 1992. A lipoxygenase pathway is activated in rice after infection with the rice blast fungus *Magnaporthe grisea*. Plant Physiol. 97:94-98.
Rathmell, W. G., and Bendall, D. S. 1971. Phenolic compounds in relation to phytoalexin biosynthesis in hypocotyls of *Phaseolus vulgaris*. Physiol. Plant Pathol. 1:351-362.
Roby, D., Broglie, K., Cressman, R., Biddle, P., Chet, I., and Broglie, R. 1990. Activation of a chitinase promoter in transgenic tobacco plants by phytopathogenic fungi. Plant Cell 2:999-1007.
Ryder, T. B., Hedrick, S. A., Bell, J. N., Liang, X., Clouse, S. D., and Lamb, C. J. 1987. Organization and differential activation of a gene family encoding the plant defense enzyme chalcone synthase in *Phaseolus vulgaris*. Mol. Gen. Genet. 210:219-233.
Samac, D. A., and Shah, D. M. 1991 Developmental and pathogen-induced activation of the *Arabidopsis* acidic chitinase promoter. Plant Cell 3:1063-1072.
Schmelzer, E., Kruger-Lebus, S., and Hahlbrock, K. 1989. Temporal and spatial patterns of gene expression around sites of attempted fungal infection in parsley leaves. Plant Cell 1:993-1001.
Schmid, J., Doerner, P. W., Clouse, S. D., Dixon, R. A., and Lamb, C. J. 1990. Developmental and environmental regulation of a bean chalcone synthase promoter in transgenic tobacco. Plant Cell 2:619-631.
Schröder, M., Hahlbrock, K., and Kombrink, E. 1992. Temporal and spatial patterns of 1,3-β-glucanase and chitinase induction in potato leaves infected by *Phytophthora infestans*. Plant J. 2:161-171.
Sequeira, L. 1976. Induction and suppression of the hypersensitive reaction caused by phytopathogenic bacteria: Specific and non-specific components. Pages 289-309 in: Specificity in Plant Diseases. R. K. S. Wood and A. Graniti, eds. Plenum Press, London.
Shibata, D., Steczko, J., Dixon, I. E., Hermondson, M., Yazdanparast, R., and Axelrod, B. 1987. Primary structure of soybean lipoxygenase-1. J. Biol. Chem. 262:10080-10085.
Shibata, D., Steczko, J., Dixon, J. E., Andrews, P. C., Hermondson, R., and Axelrod, B. 1988. Primary structure of soybean lipoxygenase L-2. J. Biol. Chem. 263:6816-6821.
Shibata, D., Shirano, Y., and Peng, Y-L. 1991. A lipoxygenase pathway for plant defense. Third International Congress of Plant Molecular Biology, (Abstracts), Tucson, Arizona, abstract No. 1295.
Shibata, D., Shirano, Y., and Peng, Y-L. 1993. Expression of a novel lipoxygenase gene is involved in plant defense against rice-blast disease. Page 350 in: Mechanisms of Plant Defense Responses. B. Fritig and M. Legrand, eds. Kluwer Academic Publishers, Dordrecht.
Slusarenko, A. J., Croft, K. P. C., and Voisey, C. R. 1991. Biochemical and molecular events in the hypersensitive response of bean to *Pseudomonas syringae* pv. *phaseolicola*. Pages 126-143 in: Biochemistry and Molecular Biology of Host-Pathogen Interactions. C. J. Smith, ed. Clarendon Press, Oxford.
Slusarenko, A. J., and Longland, A. C. 1986. Changes in gene activity during expression of the hypersensitive response in *Phaseolus*

vulgaris cv. "Red Mexican" to an avirulent race 1 isolate of *Pseudomonas syringae* pv. *phaseolicola*. Physiol. Mol. Plant Pathol. 29:79-94.

Slusarenko, A. J., Longland, A. C., and Whitehead, I. M. 1989. A convenient sensitive and rapid assay for antibacterial activity of phytoalexins. Bot. Helv. 99:203-207.

Slusarenko, A. J., Meier, B. M., Croft, K. P. C. ,and Eiben, H. G. 1993. Lipoxygenase in plant disease. Pages 211-220 in: Mechanisms of Plant Defense Responses. B. Fritig and M. Legrand, eds. Kluwer Academic Publishers, Dordrecht.

Smith, D. A., and Banks, S. W. 1986. Biosynthesis, elicitation and biological activity of isoflavonoid phytoalexins. Phytochemistry 259:79-995.

Staswick, P. E., Huang, J. F., and Rhee, Y. 1991. Nitrogen and methyl jasmonate induction of soybean vegetative storage protein genes. Plant Physiol. 96:130-136.

Stermer, B. A., Schmid, J., Lamb, C. J., and Dixon, R. A. 1990. Infection and stress activation of bean chalcone synthase promoters in transgenic tobacco. Mol. Plant-Microbe Interact. 3:381-388.

Templeton, M. D., and Lamb, C. J. 1988. Elicitors and defense gene activation. Plant Cell Env. 11:395-401.

Tranbarger, T. J., Franchesci, V. R., Hildebrand, D. F., and Grimes, H. D. 1991. The soybean 94-kilodalton vegetative storage protein is a lipoxygenase that is localized in paraveinal mesophyll cell vacuoles. Plant Cell 3:973-987.

Uritani, I., Oba, K., Kojima, M., Kim, W. K., Oguni, I., and Suzuki, H. 1976. Primary and secondary defense actions of sweet potato in response to infections by *Ceratorystis fimbriata* strains. Pages 239-252 in: Biochemistry and Cytology of Plant-Parasite Interactions. K. Tomiyama, ed. Kodanshi Ltd., Tokyo.

Voisey, C. R., and Slusarenko, A. J. 1989. Chitinase mRNA and enzyme activity in *Phaseolus vulgaris* (L.) increase more rapidly in response to avirulent than to virulent cells of *Pseudomonas syringae* pv. *phaseolicola*. Physiol. Mol. Plant Pathol. 35:403-412.

Wellmann, E., Hrazdina, G., and Grisebach, H. 1976. Induction of anthocyanin formation and enzymes related to its biosynthesis by UV light in cell cultures of *Haplopapus gracilis*. Phytochemistry 15:913-915.

Wu, R., Wu, T., and Ray, A. 1987. Adaptors, linkers and methylation. Methods Enzymol. 152:343-349.

Wyman, J. G., and Vanetten, H. D. 1982. Isoflavonoid phytoalexins and nonhypersensitive resistance of beans to *Xanthomonas campestris* pv. *phaseoli*. Phytopathology 72:483-497.

Yenofsky, R. L., Fine, M., and Liu, C. 1988. Isolation and characterization of a soybean (*Glycine max*) lipoxygenase-3-gene. Mol. Gen. Genet. 211:215-222.