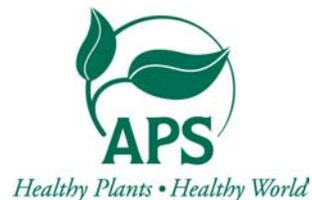This article is from the

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Stimulation of the Lipoxygenase Pathway Is Associated with Systemic Resistance Induced in Bean by a Nonpathogenic *Pseudomonas* Strain

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Systemic defense reactions induced in bean by the non-pathogenic *Pseudomonas putida* BTP1 strain reduced disease caused by *Botrytis cinerea*. Phenylalanine ammonia-lyase activity and the level of endogenous free salicylic acid were compared in plant growth-promoting rhizobacteria-treated versus control plants, but no significant differences were detected. Furthermore, no enhanced fungitoxicity was detected in methanolic leaf extracts, suggesting that accumulation of bean phytoalexins was not part of the stimulated defense mechanisms. However, BTP1-inoculated plants showed increased levels of both linoleic and linolenic acids. On this basis, we further investigated whether the lipoxygenase pathway, leading to antifungal phytooxylipins, could have been stimulated. Two key enzymatic activities of this metabolic route, namely lipoxygenase and hydroperoxide lyase, were significantly stimulated during the first four days after challenging BTP1-treated plants with the pathogen. This was observed in parallel with a more rapid consumption of the respective substrates of these enzymes, as revealed by measurements of endogenous concentrations of linolenic acid and their hydroperoxide derivatives. Moreover, headspace–gas chromatography analyses showed significantly higher concentrations of the fungitoxic final product Z-3-hexenal in leaves from BTP1-inoculated beans as compared with control plants. Taken together, these results strongly suggest that the oxylipin pathway can be associated with enhanced disease resistance induced in bean plants by nonpathogenic rhizobacteria.

Additional keyword: plant protection.

Some rhizobacteria can exert their beneficial effect on plant growth indirectly by disease suppression. Niche exclusion, competition for nutrients, siderophore-mediated competition for iron, antibiosis, and production of extracellular enzymes have been described as mechanisms involved in the disease reduction mediated by rhizobacteria (Ramamoorthy et al. 2001; Whipps 2001). Beside these direct antagonistic mechanisms, some plant growth-promoting rhizobacteria (PGPR) are also able to reduce disease through the stimulation of inducible plant defense mechanisms that render the host more resistant to further pathogen ingress. Induction of such enhanced defensive capacity can

be systemic, as root treatment with a PGPR was shown to trigger protective effects on above-ground plant parts. These reactions typically result from the activation of latent defense mechanisms that are overexpressed upon subsequent pathogen challenge (Van Loon et al. 1998). Rhizobacteria-mediated induced systemic resistance (ISR) (Kloepper et al. 1992) can occur in many plant species and was demonstrated to be effective for the control of a broad range of fungal, bacterial, and viral diseases but also against some insect and nematode pests (Van Loon et al. 1998). As the phenomenon is long-lasting and does not require extensive root colonization by the inducing microbe, ISR-based biocontrol strategies are promising, and some trials were successfully performed under field conditions (Wei et al. 1996; Zehnder et al. 2001).

ISR is phenotypically similar to the well-studied systemic acquired resistance (SAR) activated after a first infection by an incompatible necrotizing pathogen (Hammerschmidt 1999a; Sticher et al. 1997). However, the signal transduction pathway and the molecular basis underlying the rhizobacteria-mediated ISR differ in many aspects from the pathogen-induced SAR. Extensive work on *Arabidopsis thaliana* has demonstrated the dependence of ISR signal transduction on the perception of plant hormones such as jasmonate and ethylene (Pieterse et al. 2001; Ton et al. 2002), in contrast with SAR, which is characterized by an early increase in endogenously synthesized salicylic acid (SA) (Glazebrook et al. 2003; Métraux 2001). In some cases, however, enhanced levels of SA were detected in plants following treatment with PGPR. Beside SA, accumulation of pathogenesis-related (PR) proteins is also commonly associated with the expression of SAR (Van Loon and Van Strien 1999), but this phenomenon can obviously not be considered as a marker for ISR. Attempts to associate rhizobacteria-induced ISR with PR protein accumulation led to contradictory results. For instance, Maurhofer and associates (1994) showed that ISR induced by *P. fluorescens* CHAO in tobacco is coupled with stimulation of PR protein synthesis, while Hoffland (1995) and Pieterse (1996) and their associates reported that such proteins did not accumulate in radish or *A. thaliana* expressing PGPR-mediated ISR, respectively. Again, in the case of SAR, several well-characterized defense reactions, such as hypersensitive reaction (HR) (Kombrink and Schnelzer 2001), oxidative burst (Lamb and Dixon 1997), reinforcement of cell wall structures through lignification or callose deposition (Benhamou and Nicole 1999), accumulation of antimicrobial phytoalexins (Hammerschmidt 1999b; Kùc 1995), and induction of defense-related proteins with antifun-

gal properties (Linthorst 1991; Xue et al. 1998) have been extensively reported in many plant species. By contrast, protective mechanisms involved in ISR are just beginning to be elucidated. To our knowledge, HR has not yet been related to PGPR-induced resistance, and the stimulation of plant phytoalexin synthesis was only reported in *Pseudomonas*-inoculated carnation (Van Peer et al. 1991) and cucumber (Ongena et al. 2000). Only a few reports described the induction of defensive physical barriers to pathogen ingress following preinoculation with PGPR (Benhamou et al. 1996, 2000).

We have previously reported the ISR-mediated protective effect of *Pseudomonas putida* BTP1 strain on cucumber against root rot caused by *Pythium aphanidermatum* (Ongena et al. 1999). Further studies revealed that the disease protective effect was associated with a systemic increase of fungitoxic phenolics that could be considered as phytoalexins on the basis of their accumulation pattern (Ongena et al. 2000). In a recent study, we demonstrated that the same strain was also effective at reducing disease incidence caused by *Botrytis cinerea* on bean plants (Ongena et al. 2002a). Preliminary analyses of leaf extracts from bean plants treated with BTP1 revealed higher amounts of linolenic and linoleic acids as compared with control plants (M. Ongena, *unpublished data*). These polyunsaturated fatty acids serve as substrates for the lipoxygenase (LOX; EC 1.13.11.12) enzyme that initiates a metabolic route leading to the synthesis of various antifungal compounds involved in plant defenses (Blée 2002). More generally, this LOX pathway can be stimulated under different stress conditions, such as mechanical damage (Conconi et al. 1996), in response to treatment with SA (Weichert et al. 1999), methyl jasmonate (Avdiushko et al. 1995; Kohlmann et al. 1999), or biotic elicitors (Rustérucci et al. 1999) but also following interactions with pathogens (Croft et al. 1993; Montillet et al. 2002; Rancé et al. 1998). The regioselective and stereoselective dioxygenation of linoleic or linolenic acids through the LOX activity leads to the formation of the corresponding HPODE (hydroperoxy-octadecatrienoic acid) and HPOTE (hydroperoxy-octadecadienoic acid) derivatives. These highly reactive LOX products are subsequently transformed into a series of phyto-oxylipins via multiple well-characterized metabolic routes, such as i) the peroxygenase (POX) pathway leading to the conversion to fungitoxic epoxy- and hydroxy-derivatives; ii) the allene oxide synthase (AOS)

leading to the production of jasmonates known to be involved in signaling events and regulation of plant defense-gene expression; iii) the hydroperoxide lyase (HPL) forming short chain aldehydes that are believed to behave as "volatile phytoalexins", and iv) the divinyl ether synthase (DES), which has been detected only in the Solanaceae (Blée 1998, 2002; Feussner and Wasternack 2002).

This work aims to further characterize the defense mechanisms that could contribute to the enhanced level of resistance observed in bean upon treatment with strain BTP1. First, we studied some of the key enzyme activities of the LOX pathway, and the concentrations of both substrates and products were determined in order to evaluate their potential activation following treatment with strain BTP1. Special emphasis was also devoted to the analysis of volatile end-products generated by the HPL branch of this metabolism. Secondly, the possible involvement of some features commonly associated with SAR or ISR was evaluated by determining whether i) enhanced resistance triggered by BTP1 could be SA-dependent, ii) phenylalanine ammonia-lyase (PAL; EC 4.3.1.5.) activity initiating the synthesis of lignin monomers and precursors of isoflavonoid-derived phytoalexins in bean (Dixon et al. 2002) could be stimulated, and iii) accumulation of antifungal compounds in bean plant tissues could be involved.

RESULTS

Disease reduction by treatment with *P. putida* BTP1.

Botrytis cinerea infection from inoculation droplets containing spore suspension at 5×10^6 spores per ml typically resulted in brownish, spreading lesions colonizing the whole leaf area. Disease incidence was thus expressed in terms of the percentage of *B. cinerea* lesions that clearly grew out of the inoculum drop zone to produce spreading lesions. Pooled data from four independent experiments (homogeneity of variances tested by analysis of variance [ANOVA]; interactions between experiment and treatment not significant at $P = 0.05$) showed a statistically significant 34% disease reduction ($\alpha = 0.05$) in plants preinoculated at the root level with *P. putida* BTP1, as compared with the challenged controls. Figure 1 illustrates the time course of disease development in one representative experiment with disease symptoms that clearly appeared three days after pathogen challenge. As a previous study showed that the PGPR and the pathogen remained spatially separated (Ongena et al. 2002a), disease suppression was undoubtedly due to induction of resistance in the host plant.

Stimulation of lipoxygenase activity in PGPR-treated plants.

To evaluate the potential effect of BTP1 treatment on the LOX pathway, differential accumulation of linolenic and linoleic acids in infected leaf tissues was examined in three independent experiments at various times before and after pathogen challenge. Total linolenic and linoleic acids were quantified in their methylated form by gas chromatography. We observed that the C18:3 acid was the major form present in leaves, with amounts 10 times greater than those measured for the C18:2 linoleic acid (Fig. 2A and B). This reflects the ratio commonly found in leaf tissues (Montillet et al. 2002; Weichert et al. 1999) and suggests the involvement of a putative 13-LOX in bean plants, as it was previously reported (Croft et al. 1993).

Interestingly, analyses revealed twofold higher concentrations for both substrates in plants treated with the bacteria, compared with controls before pathogen inoculation. In addition, infection by *B. cinerea* resulted in a more marked decrease of fatty acid amounts in BTP1-treated leaves than in
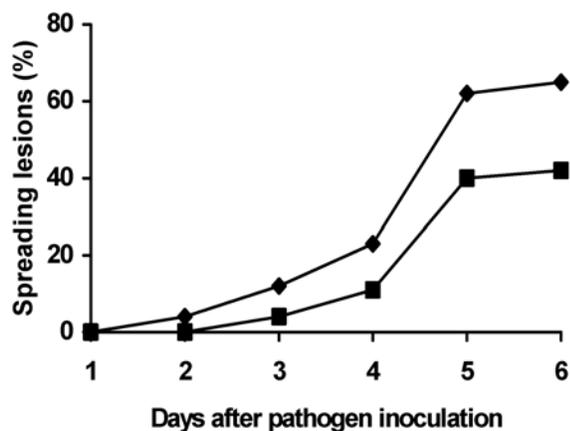

Fig. 1. Evolution of disease severity in control (♦) and BTP1-treated bean plants (■), after infection with *Botrytis cinerea*. Plants were inoculated on each primary leaf with eight 7-µl drops of a suspension (5×10^6 spores per ml) of the pathogen. Disease incidence was scored daily and was expressed in terms of the percentage of *B. cinerea* spreading lesions. Data are from one representative experiment realized with 25 plants per treatment.

controls during the first 48 h (Fig. 2A and B). This differential consumption could be related to significant increases (four-fold) in the activity of the first key enzyme of this pathway, i.e., LOX, during the same period in treated bean plants compared with controls (Fig. 2C). Starting from 48 h after pathogen challenge, LOX activity in resistant plants decreased to reach values similar to those measured in controls 5 days post-infection. This was associated with some recovery of the fatty acid concentrations during the same period.

Induction of lipid hydroperoxidase activity.

LOX introduces molecular oxygen to unsaturated linolenic and linoleic acids to yield either 9- or 13-hydroperoxides that can in turn be used by various enzymes to generate a wide variety of biologically active secondary metabolites. This global activity of all hydroperoxide-degrading enzymes is expressed as lipid hydroperoxidase activity (LHP). To further characterize the bean response, accumulation of LOX products and LHP activity were monitored. As expected on the basis of relative concentrations of linolenic and linoleic acids in leaf tissues, reversed-phase high-performance liquid chromatography (HPLC) analyses of hydroperoxides showed that HPOTE deriving from linolenic acid were prevalent. They represented more than 80% of the total lipid peroxides extracted from infected leaves in both elicited and control plants (data not shown). These analyses also revealed that the 13-isomers were largely predominant, representing up to 90% of the total HPOTE. This suggested that 13-LOX is the predominant specific activity in the leaves of bean plants, and we have therefore limited our subsequent study to the quantification of 13-HPOTE. Probably due to enhanced LOX activity in the corresponding tissues, mean values for 13-HPOTE concentrations in plants inoculated with the PGPR were about 1.7-fold higher than in water-treated controls before infection (Fig. 3A). However, as observed for fatty acids, hydroperoxide concentrations in leaves from BTP1-treated beans markedly decreased following pathogen inoculation, to reach values significantly lower than those measured in control plants 3 days after infection. These data suggested a higher consumption rate of hydroperoxides in PGPR-treated plants initiated by the perception of the pathogen.

Evolution of LHP activity was monitored by measuring fatty acid hydroperoxide-cleaving activity in leaf extracts. Prior to pathogen challenge, LHP activity in plants colonized by BTP1 was similar to that observed in noninoculated plants. However, a rapid stimulation of LHP (1.5-fold) was specifically observed in BTP1-treated plants during the first 24 h after infection (Fig. 3B). This higher LHP activity was maintained in elicited plants for 72 h. Moreover, LHP activities were also measured in extracts prepared from noninfected leaves collected in two separate experiments one day after pathogen challenge. As in infected tissues, higher LHP activities were observed in plants treated with the bacteria compared with controls with mean values (calculated from four measurements in extracts from each experiment) of 2,455 ± 124 mU per g of fresh weight (FW) and 1,632 ± 95 mU per g of FW, respectively. These data suggested that the transient induction of LHP in BTP1-treated plants was systemic and not restricted to infected organs.

Hydroperoxide lyase
as the main 13-HPOTE-degrading activity in bean leaves.

To further study the fate of fatty acid hydroperoxides and the specificity of ensuing reactions, LHP extracts of infected leaf tissues from both BTP1-treated and control plants were analyzed for the specific production of volatile aldehydes or their associated alcohols. Volatile aldehydes produced from 13-HPOTE over a period of 5 min were identified and quanti-
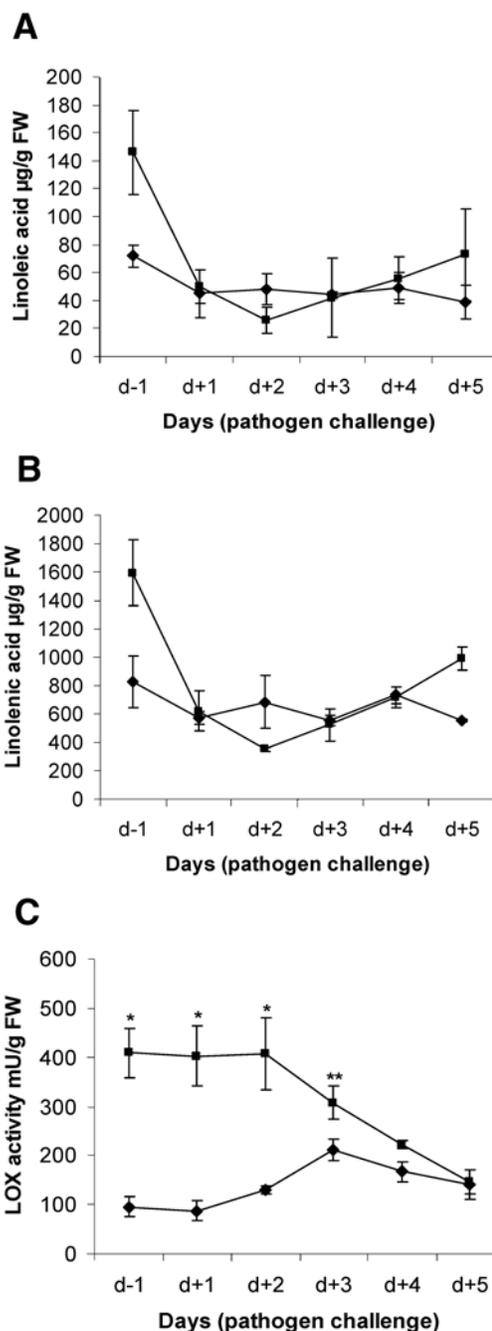

Fig. 2. Time course of lipoxygenase activity (LOX) and fatty acid substrate concentrations in bean plants previously treated with *Pseudomonas putida* BTP1 (■) and in control plants (♦). Extracts were prepared from the first true leaves collected just before inoculation of the pathogen (d-1) and 24 (d+1), 48 (d+2), 72 (d+3), 96 (d+4), and 120 h (d+5) after challenge. **A,** Concentrations of endogenous linoleic acid and **B,** linolenic acid were determined by gas chromatography after extraction with chloroform and methylation with boron trifluoride. Data are means and standard deviations calculated from three measurements performed at every sampling time in three independent experiments. **C,** LOX activities were determined on the basis of substrate consumption by enzyme extracts prepared from leaf material used for endogenous fatty acids analyses. Data are means and standard errors of four independent experiments with three measurements performed on leaf material collected at every sampling time in every experiment. Data were normally distributed and the homogeneity of variances was verified according to the Levene's test ($P = 0.05$). * and ** indicate significant differences ($\alpha = 0.001$ and 0.01, respectively) in LOX activity between controls and BTP1-treated plants at a specific timepoint, as revealed by pair-wise comparison of means with the Fisher's least significant difference test.

fied by headspace–gas chromatography (HS-GC). One main compound with retention time exactly matching (Z)-3-hexenal accumulated in the gas phase (Fig. 4). Identification of the molecule was confirmed by mass spectrometry on the basis of typical fragmentation pattern of this hexenal isomer and its

molecular ion size. GC-mass spectrometry (MS) analysis also revealed the concomitant formation of 12-oxo-(Z)-9-dodecenoic acid, the other product of 13-HPOTE-α-scission mediated by the activity of hydroperoxide lyase (HPL) (data not shown). Besides (Z)-3-hexenal, only small amounts of hexen-1-ol, directly formed from the aldehyde hexanal through the action of alcohol dehydrogenase, were detected as other HPL-derived volatile products.

HS-GC analyses of LHP products showed that the amounts of (Z)-3-hexenal generated during the 5-min incubation time were similar to the amounts of hydroperoxides consumed over the same period, as determined spectrophotometrically. This was observed in both *P. putida* BTP1-treated and control plants at day 2 or 3 postinfection in two separate experiments (Table 1). These data are consistent with HPL accounting for essentially all 13-HPOTE-degrading activity present in these leaf extracts. (Z)-3-hexenal production was about 1,350 nmol per min per g of FW and is in the same range as values of 1,300 and 3,000 nmol per min per g of FW HPL activity reported for potato (Vancanneyt et al. 2001) and tomato leaves, respectively (Fauconnier et al. 1997). Moreover, HPLC analyses at various wavelengths and GC-MS performed after methylation of reaction products did not identify any other compound that could be generated by the various HPOTE-degrading enzyme activities over the 15-min reaction time (data not shown). Incidentally, no LHP activity was observed at any time in tissue extracts containing cytosolic enzymes.
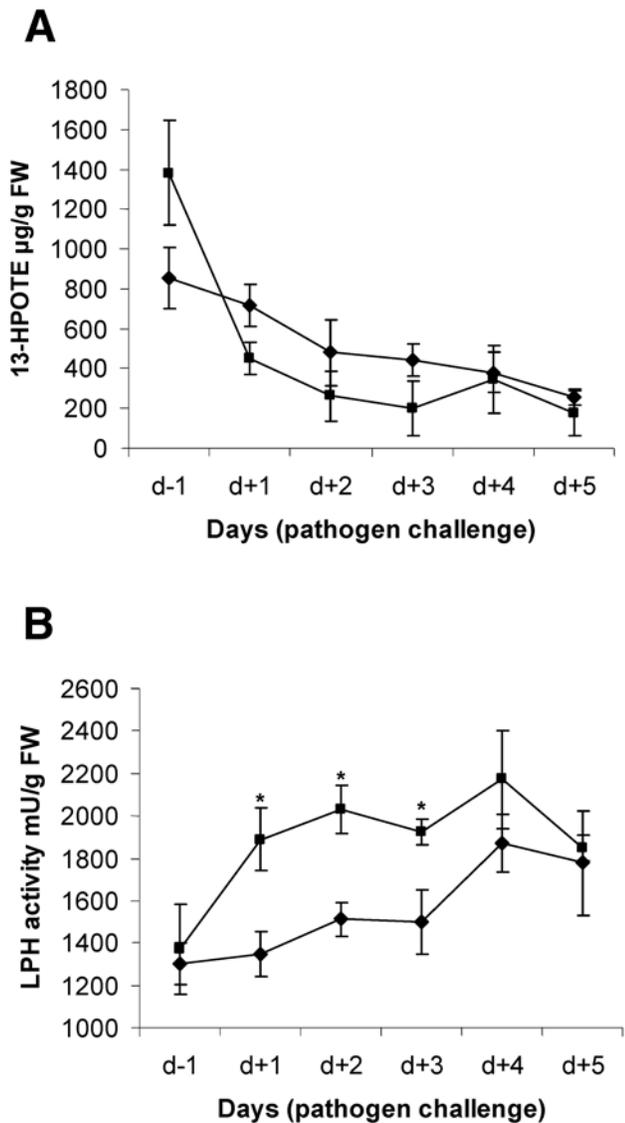
Fig. 3. Induction of lipid hydroperoxidase activity (LPH) and evolution of the 13-HPOTE (hydroperoxy-octadecadienoic acid) substrate concentration in bean plants previously treated with *Pseudomonas putida* BTP1 (■) and in control plants (♦). Extracts for both determinations were prepared from the first true leaves collected just before inoculation of the pathogen and 24 (d+1), 48 (d+2), 72 (d+3), 96 (d+4), and 120 h (d+5) after challenge. **A,** Concentration of the 13-hydroperoxide was determined by high-performance liquid chromatography after extraction with hexane/ethyl ether. Data are means and standard deviations calculated from three measurements performed at every sampling time in three independent experiments. **B,** LPH activity represents the activity of all hydroperoxide-degrading enzymes and was determined spectrophotometrically by monitoring hydroperoxide decomposition at 234 nm. Enzyme extracts were prepared from leaf material used for endogenous hydroperoxide concentration analyses. Data are means and standard errors of four independent experiments with three measurements performed on leaf material collected at every sampling time in every experiment. Data were normally distributed, and the homogeneity of variances was verified according to the Levene's test ($P = 0.05$). * indicates significant differences ($\alpha = 0.05$) in HPL activity between controls and BTP1-treated plants at a specific timepoint, as revealed by pair-wise comparison of means with the Fisher's least significant difference test.
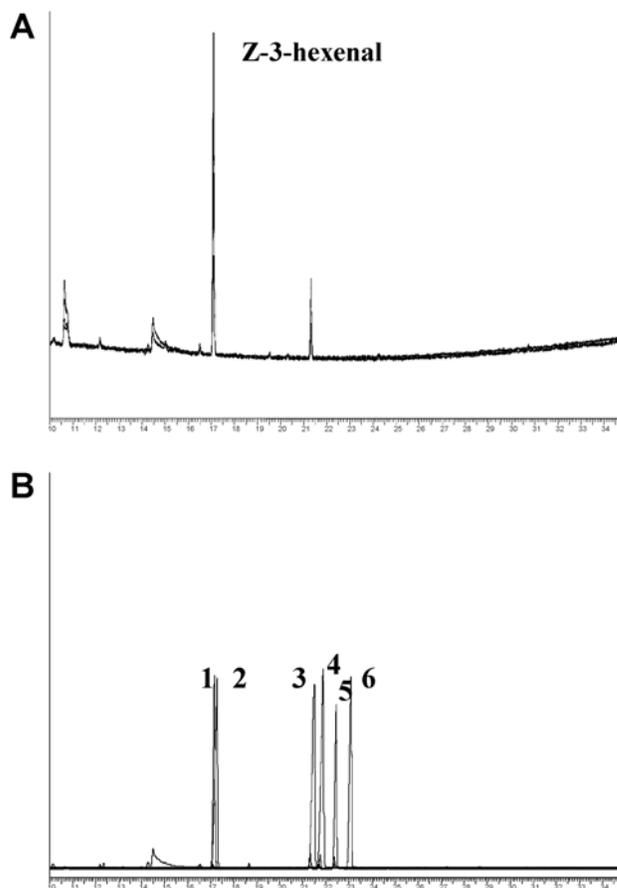
Fig. 4. Typical headspace–gas chromatography profile resulting from the **A,** analysis of volatile aldehydes present in bean leaves compared to the **B,** chromatogram obtained for analysis of a mixture of standards: 1, z-3-hexenal; 2, E-2-hexenal; 3, hexen-1-ol; 4, E-3-hexenol; 5, Z-3-hexenol; 6, E-2-hexenol.

Accumulation of hexenal in PGPR-treated plants.

HPL thus appeared to be highly active in bean leaves, and we therefore evaluated the abundance of HPL-derived volatile aldehydes in these infected tissues by HS-GC. Although traces of (E)-2-hexenal, (E)-2-hexenol, and two other unidentified volatile aldehydes were also detected; co-chromatography with authentic standards and GC-MS analyses revealed that the 13-HPOTE-derived metabolite (Z)-3-hexenal was again the major derivative (data not shown). Root treatment with *P. putida* BTP1 also resulted in a transient increase in the amount of (Z)-3-hexenal in bean leaves. Concentrations of this C_6-aldehyde were similar in control and BTP1-treated plants, before infection with *B. cinerea*. However, during the first two days after pathogen challenge, significantly higher concentrations were observed in leaves of plants inoculated with BTP1 (Fig. 5). After 48 h, this aldehyde accumulated to 25 nmoles per g of FW in treated beans, an increase of about 40% as compared with control plants.

Table 1. HPL and LPH activities in bean leaves treated or not treated with *Pseudomonas putida* BTP1

Type of plant[a]	Z-3-hexenal produced (nmol/min)[b]	13-HPOTE consumed (nmol/min)[c]
Control (2)	110 ± 12[d]	106 ± 17
BTP1-treated (2)	243 ± 26	247 ± 40
Control (3)	153 ± 17	170 ± 43
BTP1-treated (3)	336 ± 37	354 ± 56

[a] Followed by the number of days after pathogen challenge. Assays were realized on tissues from the first true leaves collected 48 or 72 h after pathogen challenge.
[b] Represents hydroperoxide lyase activity (HPL) activity based on the direct analysis of formed aldehydes by headspace-gas chromatography.
[c] Represents lipid hydroperoxidase activity (LPH) activity based on the spectrophotometric measurement of hydroperoxide decomposition of absorbance at 234 nm. HPOTE = hydroperoxy-octadecadienoic acid.
[d] Mean ± standard error of three replicates per treatment.
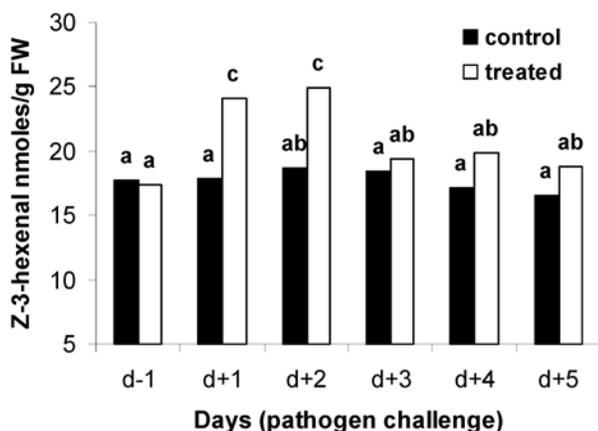

Fig. 5. Evolution of endogenous Z-3-hexenal concentrations in bean leaves from control and BTP1-treated plants before pathogen inoculation (d-1) and 24 (d+1), 48 (d+2), 72 (d+3), 96 (d+4), and 120 h (d+5) following the challenge. Z-3-hexenal was analyzed by headspace–gas chromatography in infected leaf samples randomly collected in each treatment from three plants at every sampling time (pooled material). Data are means calculated from four independent experiments with three measurements performed on leaf material collected at every sampling time in every experiment. Data were normally distributed, and the homogeneity of variances was verified according to the Levene's test ($P = 0.05$). Bars with the same letters are not significantly different ($\alpha = 0.05$), according to pair-wise comparison of means with the Fisher's least significant difference test.

ISR is not associated with endogenous SA accumulation, PAL stimulation, or higher level of phytoalexins.

Endogenous SA was assayed in leaf tissues collected two days after pathogen challenge in two separate experiments. However, no significant difference was observed in the amounts of either free or conjugated SA recovered from bacterized plants, with values of 99 ± 60 and 3,310 ± 320 ng per g of FW, respectively, as compared with controls with 93 ± 56 and 2,910 ± 510 ng per g of FW, respectively.

We also examined whether PAL activity could actually be stimulated in plants bacterized with *P. putida*. Globally, no clear differences were observed between treated and control plants before infection and during the first 5 days after pathogen challenge (Fig. 6). A transient increase was noted at day 3 postinfection, but it is difficult to associate such a limited stimulation with any metabolic change leading to a strong and durable defense response.

In an attempt to correlate the protective effect observed upon treatment with *P. putida* BTP1 with specific accumulation of antifungal compounds in bean plant tissues, methanolic extracts from infected and noninfected leaves were analyzed for their fungitoxic activity. The extraction procedure recovers typical bean phytoalexins such as phaseolin, phaseolidin, and phaseolinisoflavan (Hynes et al. 1994). No marked fungitoxicity could be visualized at any time in extracts from either BTP1-treated plants or control plants, either in thin-layer chromatography bioassay against *Cladosporium cucumerinum* or in agar diffusion-based tests for growth inhibition of *B. cinerea* (data not shown).

DISCUSSION

Our results demonstrate that treatment of bean roots with *Pseudomonas* strain BTP1 conferred some decreased susceptibility against gray mold caused by *B. cinerea*. This disease reduction was clearly associated with the stimulation of the activities of two key enzymes involved in the LOX pathway, i.e., lipoxygenase and hydroperoxide lyase. LOX activity appeared to be enhanced in noninfected plants following PGPR treatment, but higher levels were also maintained for 2 days after challenge with the pathogen. At this time, no apparent tissue necrosis or
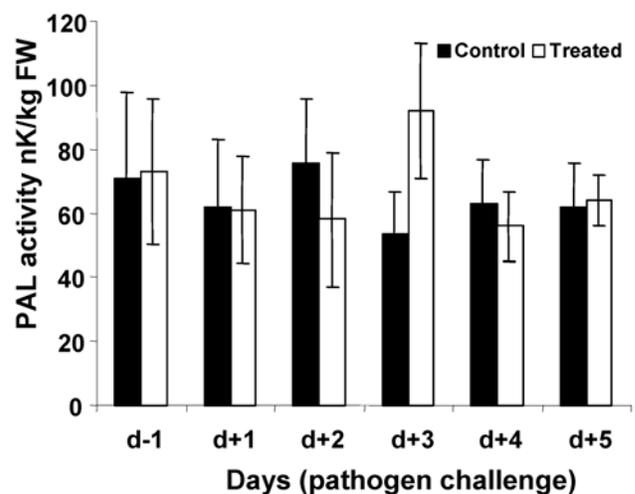

Fig. 6. Time course evolution of phenylalanine ammonia-lyase (PAL) activity in leaves of bean plants treated or not treated with *Pseudomonas putida* BTP1. PAL activity measurements were performed on infected leaf material collected 24 (d+1), 48 (d+2), 72 (d+3), 96 (d+4), and 120 h (d+5) after pathogen challenge or just before infection (d-1). Data are means and standard deviations calculated from two measurements of samples collected at every timepoint in two independent experiments.

typical symptoms associated with hypersensitive cell death could be visualized on leaves. This suggests that the observed LOX induction did not rely on any wounding response phenomenon, nor was it associated to some hypersensitive response development, as demonstrated in incompatible plant-pathogen interactions (Montillet et al. 2002). The increased lipid peroxidation observed before infection, illustrated by both higher LOX activity levels and higher concentrations of endogenous hydroperoxides, thus appeared to be specifically induced upon perception of *P. putida* BTP1 by root cells.

In contrast to LOX, similar hydroperoxide lyase activities were observed in both BTP1-treated and control bean plants before infection. However, the activity of this enzyme appeared to be significantly enhanced in plants inoculated with BTP1 in the first 24 h following infection with the pathogen. This early HPL stimulation may explain why no accumulation of endogenous hydroperoxides could be observed at any time postinfection, despite higher LOX levels in BTP1-treated plants. As a result of enhanced HPL activity, significantly higher amounts of Z-3-hexenal were found in leaves from BTP1-treated plants compared with controls during the first two days after inoculation with the pathogen. These C6 aldehydes are among the most antifungal lipid-derived products generated by HPL activity, and they retain some toxicity against various fungal plant pathogens (Deng et al. 1993; Vaughn and Gardner 1993). Hexenal concentrations in leaves from control plants did not vary significantly over the sampling time and were seemingly not sufficient to readily inhibit pathogen ingress. However, hexenal amounts increased by 40% in BTP1-treated plants compared with controls. This differential accumulation of approximately 8 nmoles per g of FW is in the range of the quantities previously reported by Hamilton-Kemp and associates (1992) to be inhibitory to the development of *B. cinerea*. These authors showed that fungal growth was strongly impaired when spores have to germinate in an environment containing hexenal in trace amounts as low as 20 nmoles per liter of air. Moreover, in our experiments, it is possible that these values measured at the specific time of sampling in leaf tissues do not reflect the amounts that are actually produced. Previous experiments on bean have shown a very effective release of these compounds in the atmosphere at rates ranging from 5 to 20 nmoles per hour per pair of first leaves (Croft et al. 1993). With such large amounts being lost from leaves, it seems fair to assume that the quantities effectively produced at sources within the leaf tissues might be in the range that could locally (e.g., in the intercellular spaces) restrict hyphal penetration of *Botrytis*. These molecules could, therefore, play a role as "volatile" phytoalexins, as previously suggested in the interaction of bean with *Pseudomonas syringae* by Croft and associates (1993).

The timing of hexenal accumulation and, more generally, of changes in the LOX pathway induced upon treatment with the bacterial strain is interesting with regard to disease development. The early accumulation of antifungal volatile products in infected leaves during the first 48 h after pathogen challenge most probably corresponds either to the period of spore germination, the early stage of pathogen growth, or both, leading to only few spreading lesion symptoms. Thus, it is obvious to postulate that higher levels of hexenal in treated plants could somewhat restrict this early stage of *Botrytis* development, as illustrated by the delay observed for the appearance of spreading lesions in BTP1-treated plants compared with controls (Fig. 1). Hamilton-Kemp and associates (1992), who reported the antifungal activity of hexenal, also used fungal spores to test the toxicity of the molecule, leading to the conclusion that hyphal reduction was mainly due to an inhibitory effect on spore germination.

Interestingly, beside their fungitoxic effect, C_6-volatiles derived from the LOX pathway also have some defense-related gene-induction activity, as observed in various plants upon treatment with methyl jasmonate (Avdiushko et al. 1995). Experiments conducted on *A. thaliana* showed that the presence of these hexenals and associated alcohols clearly induced the activity of enzymes involved in the oxylipin pathway, i.e., LOX and AOS (Bate and Rothstein 1998). Such a positive feedback regulation of oxylipin synthesis by C_6 aldehydes could also occur in BTP1-treated plants. By contrast, expression of PAL was either not stimulated or was stimulated to a much lower extent by the presence of volatiles in *A. thaliana*, nor was the accumulation of PR-1 and PR-2 proteins or the defense-related thionin stimulated (Bate and Rothstein 1998). This is in line with the lack of PAL induction observed in bean upon treatment with BTP1.

The stimulation of HPL and the accumulation of fungitoxic volatile products were only detected when bacterized plants were challenged with *B. cinerea*. This observation supports the concept that a signal produced by the pathogen is essential for triggering enhanced synthesis and accumulation of defense gene products (Benhamou 1996; Van Loon et al. 1998). In line with this concept, Van Peer and associates (1991) reported that enhanced phytoalexin accumulation occurred in *Pseudomonas*-treated carnation roots at the onset of pathogen attack. Previous studies in cucumber plants treated with BTP1 also showed a pathogen-dependant systemic accumulation of fungitoxic compounds, most probably released from a pool of conjugated phenolics (Ongena et al. 2000). Benhamou and Nicole (1999) also demonstrated that the induction of structural and biochemical cellular barriers in bacterized cucumber seedlings was much higher when roots were challenged with *Pythium ultimum*. Such a pathogen-dependent enhanced expression of host defense mechanisms appears to be a common feature of ISR induced by PGPR in plants.

The onset of ISR is generally not associated with major changes in gene expression, and some of the defense-related genes identified in the case of pathogen-induced SAR were not found to be up-regulated. However, some studies have associated the protective effect with specific host metabolic changes in plants developing PGPR-induced resistance. In several plant species, such as tomato (M'Piga et al. 1997), cucumber (Benhamou et al. 2000), or pea (Benhamou et al. 1996), microscopy studies have provided direct evidence for a deposition of phenolic compounds that reinforce the cell wall structure upon polymerization (lignin). These phenolics derived from the phenylpropanoid metabolism initiated by the PAL activity can also contribute to the creation of a fungitoxic environment around the sites of penetration of the pathogen. As this enzyme activity is not stimulated in BTP1-treated plants, it is unlikely that lignification plays a crucial role in the protection. Another prominent feature of SAR is the stimulation of antifungal phytoalexin synthesis (Kùc 1995). However, reports about this phenomenon and its role in PGPR-mediated ISR are still limited (Ongena et al. 2000; Van Peer et al. 1991; Zdor and Anderson 1992). As the phenylpropanoid pathway also provides precursors of isoflavonoid-derived phytoalexins in bean (Dixon et al. 2002), the lack of PAL induction upon BTP1 treatment also suggests that these compounds are not involved in plant protection provided by this bacterium. This was supported by the absence of marked fungitoxicity in methanolic extracts from both BTP1-treated plants and controls. However, in cucumber, ISR provided by BTP1 was associated with the stimulation of a systemic response based on the accumulation of antifungal phytoalexins (Ongena et al. 2000). This further exemplifies the strong heterogeneity in the nature of defense reactions inducible by one particular PGPR strain in different plant species (Benhamou et al. 1996; Van Loon et al. 1998).

Some nonpathogenic rhizobacteria induce a SAR-type defense response associated with either endogenous free SA accumulation, PR protein expression, or both (Maurhofer et al. 1998; Timmusk and Wagner 1999). De Meyer and associates (1999a and b) also demonstrated the potential of *Pseudomonas aeruginosa* 7NSK2 derivatives to induce the SAR pathway in tobacco and bean. In these studies, induced resistance was associated with early induction of PAL activity, a major enzyme in SA synthesis (Pallas 1996), followed by an accumulation of free SA in the leaves. In our work, using the same bean-*Botrytis* pathosystem, *P. putida* BTP1 failed to initiate either any significant SA accumulation or any durable stimulation of PAL, suggesting that the resistance is SA-independent. ISR induced by BTP1 probably derives from molecular events associated with the response depending on jasmonate, synthesized from 13-hydroperoxide via the allene oxide synthase branch, and ethylene (Pieterse et al. 2001). However, HPLC analyses of leaf extracts from BTP1-treated beans did not reveal any increase in the endogenous concentrations of jasmonic acid or of its methylated form (data not shown). This is in line with previous observations suggesting that the ISR transduction pathway involves an increase in jasmonic acid sensitivity rather than an accumulation of this hormone in plant tissues (Pieterse et al 2001).

In conclusion, this study showed the ability of a nonpathogenic fluorescent *Pseudomonas* strain to stimulate a systemic LOX-dependent response in bean that was associated with increased protection against *B. cinerea* infection. Lipid peroxidation or subsequent reactions, or both, leading to the synthesis of oxylipins were demonstrated to be involved in defense and stress reactions and are inducible by various physical, chemical, and biotic factors (Blée 2002). To our knowledge, this work provides the first example of a selective induction of LOX and HPL enzymes as a response to treatment with PGPR. We hypothesize the sequential activation of these two key enzymes by root preinoculation with BTP1. The bacterium sensitized bean plants to react more efficiently to subsequent pathogen infection. This led to the accumulation of antifungal volatile products that could act locally to restrict pathogen ingress. However, hexenal accumulation should be considered as one component of the bean response. There is a multitude of oxylipins derived from other branches of the LOX pathway that display various biological activities and, thus, could play crucial roles in the plant defense process. By analogy with the pathogen-induced SAR, macroscopic disease reduction triggered by PGPR treatment may result from a biochemically complex and multicomponent response leading to the concomitant stimulation of several defense mechanisms with additive effects. Whether some other plant responses, such as production of lytic enzymes with antifungal activities or plant cell wall reinforcement, are stimulated or not in bean elicited by strain BTP1 remains to be elucidated.

MATERIALS AND METHODS

Microbial strains.

P. putida BTP1, isolated from barley roots, was originally selected for its specific features regarding pyoverdine-mediated iron transport (Jacques et al. 1995; Ongena et al. 2002b). It was maintained on Pseudomonas Agar F medium (Becton, Dickinson and Company, Le pont de Claix, France) at 4°C, before experimental use and was stored at –80°C in cryotubes, according to manufacturer recommendations (Microbank, Prolab Diagnostic, Richmond Hill, Canada), for long-term conservation. The fungal pathogen *Botrytis cinerea* was provided by M. Höfte (Ghent University) (De Meyer et al. 1997). *B. cinerea* was routinely grown to sporulation on an oat-based medium (oatmeal 25 g/liter; agar 12 g/liter), at room temperature. For long-term storage, spores were conserved in 40% glycerol at –70°C.

Assays for induced resistance.

ISR assays were performed with bean (*Phaseolus vulgaris*) cv. Prelude, following a procedure previously described (Ongena et al. 2002a). Plants were grown either in a peat substrate (Brill Substrate GmbH & Co KG, Georgsdorf, Germany) referred to as "soil" or in a perlite-vermiculite mix (50:50, vol/vol). Prior to planting, beans were disinfected in $HgCl_2$ 0.1% for 7 min, were rinsed three times in sterile distilled water, and were soaked for 10 min in a bacterial suspension at a concentration of approximately 4×10^8 CFU ml^{-1} or, in the case of control plants, in NaCl 0.85%. Bean seeds were then sown in 10-cm pots containing a peat substrate (Brill Substrate GmbH & Co KG) compost soil or perlite-vermiculite previously mixed with bacterial inoculum to a final concentration of 3×10^7 CFU g^{-1} or with an equal volume of sterile water for untreated control plants. Beans were germinated at $22 \pm 2°C$ in the greenhouse with a 16-h photoperiod. Seven and 14 days after sowing, 20 ml of a bacterial suspension at 10^8 CFU ml^{-1} were added as a drench to the roots of each plant except the controls (watered with 20 ml of NaCl 0.85%). After approximately 20 days, bean plants were transferred to a high humidity chamber ($19 \pm 2°C$). After 24 h, they were leaf-infected with *B. cinerea*. To this end, eight wounds were made on both primary leaves by gently touching the epidermis with a red hot pinhead. These wounds were covered with 10 μl of the pathogen spore suspension. After 4 to 5 days, the disease incidence was expressed in terms of the percentage of *B. cinerea* lesions that clearly grew out of the inoculum drop zone to produce spreading lesions. Briefly, sterilized seeds were dipped for 10 min in a bacterial suspension at a concentration of 4×10^8 CFU/ml, prior to sowing. Bean plants were then grown in potting soil previously mixed with BTP1 cells to a final concentration of 3×10^7 CFU/g. Additionally, 6 days after sowing, 20 ml of a bacterial suspension at 10^8 CFU/ml were added as a drench to the roots of each plant. After approximately 16 days, bean plants were transferred to a high-humidity chamber ($19 \pm 2°C$) for 24 h before leaf infection with *B. cinerea*. This was achieved by inoculating each primary leaf with eight 7-μl drops of a suspension at 5×10^6 spores ml^{-1}, prepared as described (Ongena et al. 2002a). In every experiment, 25 plants were used per treatment. Disease incidence was scored daily and was expressed in terms of the percentage of *B. cinerea* lesions that clearly grew out of the inoculum drop zone to produce spreading lesions. Experiments were carried out four times and contained 25 plants per treatment. For disease reduction evaluation, the homogeneity of variances was tested by ANOVA, and data from different experiments with the same set-up were pooled for mean comparison between the two treatments (least significant difference [LSD] at $\alpha = 0.05$), as the ANOVA revealed that interaction between experiment and treatment was not significant at $P = 0.05$. Means from the different treatments were compared by the method of Newman and Keuls (LSD at $\alpha = 0.05$).

In every experiment, leaves from three plants within the same treatment were randomly harvested just before challenge with the pathogen (d-1) and 24 h (d+1), 48 h (d+2), 72 h (d+3), 96 h (d+4), and 120 h (d+5) after infection. At every sampling time, leaves from the three individual plants were pooled and immediately frozen in liquid nitrogen and were reduced to a fine powder with mortar and pestle. Ground leaf tissues were kept at –25°C until further use for enzyme or chemical analyses.

Measurement of salicylic acid.

Free and bound SA were extracted and measured by HPLC (Hewlett-Packard 1100 series system, Agilent Technologies, Waldbronn, Germany), following the method described by Meuwly and Métraux (1993) with some modifications. Extractions were performed on 1-g samples of ground leaf tissue obtained as described above, and leaf extract samples (20 μl) were injected onto a Inertsil 5 ODS-2 (250 × 3 mm) column (Varian-Chrompack, Middelburg, The Netherlands) and were eluted at a flow rate of 0.55 ml/min at room temperature. Under such conditions, SA had a retention time of 16.3 min. It was quantified by reference to the standard curves established with commercial SA at various concentrations. SA analysis was repeated five times for each treatment, and results presented are means and standard deviations calculated from pooled data from two independent experiments.

Determination of PAL activity.

PAL activity in bean leaves was determined spectrophotometrically by measuring the conversion of L-Phe to trans-cinnamate (De Meyer et al. 1999b). Samples of leaf tissue powder were extracted with 50 mM Tris-HCl (pH 8.5) containing 14 mM mercapto-ethanol and 5% (wt/vol) polyvinylpyrrolidone. Extracts were incubated for 1 h on ice and were homogenized by vortexing every 10 min. Homogenates were then centrifuged 5 min at $13,000 \times g$ at 4°C, and the resulting supernatants were used for PAL activity determination at 40°C in 3 ml of 50 mM Tris-HCl (pH 8.5) supplemented with 10 mM phenylalanine. The evolution of the optical density at 290 nm was monitored with a UVIKON XS spectrophotometer (Beun-De Ronde Serlabo sa, Drogenbos, Belgium) for 1 h, and the amount of trans-cinnamic acid generated was calculated using an ε of 11,600 $M^{-1}cm^{-1}$. PAL activity measurements were performed twice with leaf material collected at every timepoint in two independent experiments.

Antifungal activity of leaf extracts.

Methanolic extracts of ground leaf tissues were prepared from samples consisting of 2 g of frozen material, as described previously (Ongena et al. 2000). Their fungitoxic activity was tested against *Cladosporium cucumerinum* after thin-layer chromatography on silica gel plates, based on the method of Daayf and associates (1995). In this test, zones of toxicity appear as white spots against a dark gray background that developed after fungal growth. The fungal growth inhibition potential of the leaf extracts was also tested against *B. cinerea* on plate bioassays. Aliquots (80 μl) of individual extracts and of methanol (control) were dispensed in wells made in the oat-based medium. The surface was then homogeneously covered by *B. cinerea* spores by plating 100 μl of a suspension at 1.5×10^6 spores/ml. Plates were incubated at 25°C for 72 to 96 h before observation of putative growth inhibition in the zone of diffusion around the wells.

**Determination
of endogenous fatty acids and hydroperoxides.**

The procedure used for linoleic and linolenic acid extraction was adapted from the method described by Conconi and associates (1996). Samples consisting of 0.25 g of frozen powder prepared from control or treated plants were suspended in 1 ml of chloroform/methanol/formic acid/water (10:10:1:1, vol/vol/vol/vol) and were stored overnight at –20°C. Samples were then centrifuged for 5 min at $13,000 \times g$, and the tissue pellet was re-extracted with 1 ml of chloroform/methanol/water (5:5:1, vol/vol/vol). After incubation for 90 min at 4°C followed by centrifugation, the two supernatants were combined and washed with 0.5 ml of 0.2 M H_3PO_4, 1 M

KCl. The chloroform phase was recovered and was evaporated in a speed-vac concentrator. Fatty acids were dissolved in 200 μl of hexane and were methylated by adding 0.5 ml of 12% BF3/methanol/hexane (2.5:55:2, vol/vol/vol) at 70°C for 90 min in screw-capped tubes. Water (400 μl) and hexane (300 μl) were then added to 200 μl of the reaction products. Fatty acid methyl esters present in the hexanic phase were separated by gas chromatography (Hewlett-Packard 5890 series II apparatus, Agilent Technologies) on a CPWAX 52 CB column (50 m × 0.32 mm, Varian-Chrompack) with flame ionization detection. The oven temperature was programmed for 5 min at 180°C followed by a 4°C min^{-1} ramp to 220°C and a second ramp of 6°C min^{-1} to 250°C and was maintained at 250°C for an additional 5 min. The methyl ester fatty acid peaks were quantified and identified by comparison of their retention times (i.e., 22.5 and 24.3 min for methyl linoleic acid and methyl linolenic acid, respectively) with those of commercial standards.

Hydroperoxides were extracted from leaf tissues of control and treated plants by adding 1 ml of hexane to 0.25-g samples of frozen tissue powder. Extracts were incubated for 90 min in the dark at 25°C before centrifugation for 5 min at $13,000 \times g$. The pellet was then re-extracted with 1 ml of hexane/ethyl ether (50:50, vol/vol) under the same conditions and was centrifuged. The two supernatants were combined and evaporated to dryness under vacuum in a speed-vac concentrator. The residue was resolubilized in 200 μl of methanol, and this solution was analyzed by reversed-phase HPLC (HP 1100 series system) on a LiChrospher 100 RP C-18 column (250 × 4.6 mm, 5 μm packing; Merck, Darmstadt, Germany), using a solvent system of acetonitrile/water/20 mM Na_2HPO_4 at a constant flow rate of 0.7 ml/min. Hydroperoxides were eluted with a gradient of acetonitrile as follows: (time in min/% acetonitrile/% water/% 20mM Na_2HPO_4, in volume) 0:45:45:10, 22:45:45:10, 42:53:37:10, 60:53:37:10, 70:85:5:10, 75:90:0:10. Absorbance at 234 nm indicating the conjugated diene was recorded. Under these conditions, 13-HPOTE were separated from the 9-isomers (retention time of 42.2 min) and were eluted after 43.5 min. Hydroperoxides were identified and quantified on the basis of retention times, and calibration curves were established with synthetic compounds used as standards (discussed below). The extraction product was thus used for HPL activity assays without further purification. HPOTE were quantified on the basis of absorbance at 234 nm, using an extinction coefficient of 25,000 $M^{-1} cm^{-1}$.

Preparation of LOX and HPL crude extracts.

For LOX extraction, 0.5 g of frozen leaf tissue powder was added to 3 ml of ice-cold 0.1 M sodium phosphate buffer containing 0.4 g $Na_2S_2O_5$ per liter and 2.5 g of Tween 20 per liter. This mixture was incubated for 1 h on ice and was homogenized by vortexing every 15 min. It was then centrifuged for 15 min at $20,000 \times g$ at 4°C, and the supernatant was used as crude extract for the LOX assay. The same procedure was applied for HPL activity extraction, except that 0.1% Triton X-100 was added to the extraction buffer.

LOX activity assays.

LOX activity was determined on the basis of substrate consumption. The crude enzyme extract prepared as described above (50 μl) was added to 2.940 ml of oxygenated 0.1 M sodium phosphate buffer, pH 7, and 10 μl of 18 mM linolenic acid in NaOH 0.05 N. The reaction was stopped by lowering the pH to a value of 3 with 6 N HCl either immediately or after a 2-min incubation time at 30°C. Aliquots (80 μl) were then submitted to reverse-phase HPLC without further purification for initial and residual linolenic acid concentration measurements. Samples were injected on the LiChrospher 100

RP C-18 column and were eluted isocratically at a constant flow rate of 1 ml min^{-1} with acetonitrile/water/acetic acid 82:18:0.1 (vol/vol/vol). Under these conditions, linolenic acid eluted as a single peak after 7.9 min (UV detection at 205 nm) and was quantified using calibration curves established by solubilizing various amounts of pure linolenic acid in the same sodium phosphate buffer. In every experiment, three measurements for LOX activity were realized on leaf material collected at every sampling time (discussed above). Statistical analysis was performed on pooled data from four independent experiments, using STATISTICA software (StatSoft, Inc., Maisons-Alfort, France). Data were normally distributed, and the homogeneity of variances was verified according to the Levene's test ($P = 0.05$). One-way ANOVA revealed significant differences between the treatments ($P = 0.05$), and further pair-wise comparison of means obtained for each treatment at every timepoint was realized by the Fisher's LSD test ($\alpha = 0.05$ or 0.01).

Substrate synthesis and HPL activity assays.

13-HPOTE was synthesized using commercial soybean lipoxygenase and was extracted as described by Fauconnier and associates (1997). The purity of reaction products was assessed by HPLC with the method mentioned above. The extraction product was thus used for HPL activity assays without further purification. HPOTE were quantified on the basis of absorbance at 234 nm, using an extinction coefficient of 25,000 M^{-1} cm^{-1}.

Two methods were used for the determination of HPL activity in leaves of bean plants. In the spectrophotometric assay, the activity of all hydroperoxide-degrading enzymes is determined and globally expressed as lipid hydroperoxidase activity (LHP). A typical assay consisted of 2,940 μl of 0.1 M sodium phosphate buffer, pH 6.8, 7 μl of 8 mM 13-HPOTE in NaOH 0.05 N, and 50 μl of crude extract obtained as described above. Hydroperoxide decomposition was monitored by following the decrease in absorbance at 234 nm for 60 s, and one unit of LHP activity was expressed as the amount of enzyme consuming 1 μmol of 13-HPOTE in 1 min. This method was used for measurements of HPL activity in leaf material from control and treated plants collected at the various sampling times in four independent experiments. Data set and statistical analysis were as described for LOX.

The second method is more specific for HPL activity and is based on the direct analysis of formed aldehydes by HS-GC. For this purpose, a similar enzymatic reaction was carried out in sealed 15-ml glass vials for 1 min and was stopped by lowering the pH to 3 with 12 N HCl. The vials were then transferred into an automatic headspace sampler (HS 40 XL, Perkin Elmer, Norwalk, CT, U.S.A.) and were incubated at 80°C for 15 min, to allow the produced aldehydes to enter the gas phase. Volatile samples were then automatically injected onto a fused silica CP-WAX 52CB column (50 m × 0.32 mm id; Alltech Associates, Deerfield, IL, U.S.A.) in the gas chromatograph (Autosystem XL, Perkin Elmer) equipped with a flame ionization detector. Oven temperature gradient was set as follows: 0 to 5 min, 50°C; 5 to 40 min, from 50 to 190°C, at a rate of 4°C per min. Z-3-hexenal formed was quantified by injection of various amounts of the pure compound (Fontarome Chemical, Inc., St. Francis, WI, U.S.A.). The identity of the aldehyde was confirmed by GC-MS analysis (Fauconnier et al. 2003) of a hexanic extract prepared from the reaction medium (extracted twice with 3 ml of n-hexane).

Determination of endogenous C-6 aldehydes.

Endogenous HPL-derived volatiles in bean were quantified from ground leaf tissue. Samples of frozen plant material (1 g)

were placed in 12-ml headspace vials containing 3 ml of 200 mM sodium phosphate buffer, previously adjusted to pH 3 with 12 N HCl. Vials were immediately sealed and were submitted to both quantitative and qualitative analyses by HS-GC and GC-MS, respectively, as described above for the HPL assay. In every experiment and at every sampling time, three samples consisting of pooled leaf material collected from different plants, as described above, were analyzed for their hexenal content. Statistical analysis was performed on pooled data from four independent experiments, as described for LOX.

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