Induction of Hydroxycinnamoyl-Tyramine Conjugates in Pepper by *Xanthomonas campestris*, a Plant Defense Response Activated by *hrp* Gene-Dependent and *hrp* Gene-Independent Mechanisms

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Inoculation of pepper leaves, *Capsicum annuum* cv. Early Calwonder ECW 10R, with strains of *Xanthomonas campestris* led to an accumulation of the phenolic conjugates feruloyltyramine (FT) and *p*-coumaroyltyramine (CT) 24 h postinoculation in nonhost- and gene-for-gene-determined incompatible interactions with *X. campestris* pv. *campestris* and *X. campestris* pv. *vesicatoria*, respectively. In contrast, neither compound was detected in compatible interactions with *X. campestris* pv. *vesicatoria*. The accumulation of FT and CT was preceded by an increase in the extractable activity of tyrosine decarboxylase as well as increases in the transcription of genes encoding phenylalanine ammonia-lyase and tyramine hydroxycinnamoyl transferase. No such changes were detected in compatible interactions. Very rapid accumulation of FT and CT occurred (4 h postinoculation) in pepper in response to a *X. campestris* pv. *campestris* mutant carrying a deletion of the *hrp* gene cluster. In contrast, *hrp* mutants of *X. campestris* pv. *vesicatoria* failed to elicit the production of FT and CT. These observations suggest the existence of *hrp* gene-dependent and -independent activation mechanisms of a defense response involving hydroxycinnamoyltyramines.

Additional keywords: antibacterial compounds; *avrBsI* avirulence gene; *BsI* resistance gene.

Plant resistance to microbial attack is associated with the activation of a number of plant responses that are triggered following recognition by the plant cell of molecules of pathogen origin. In the area of plant responses to bacterial pathogens, interactions explained genetically by the gene-for-gene hypothesis have received the most attention. Recognition of a bacterial avirulence protein by its cognate resistance gene

product is believed to lead to the activation of a number of defense-related responses, including the hypersensitive response (HR) (Hammond-Kosack and Jones 1997). It is thought that these avirulence proteins are delivered into the plant cell via a type III secretion system encoded by bacterial genes within the *hrp* gene cluster (for hypersensitive resistance and pathogenicity) (Bonas and Van den Ackerveken 1997; Lindgren 1997).

Mechanisms of induction of defense-related responses that are *hrp* independent also exist, but have received far less study. These effects have been termed “nonspecific” because they do not require the recognition of specific avirulence gene products by the plant (Jakobek and Lindgren 1993). Additionally, Jakobek and Lindgren (1993) have shown that a *hrp* mutant of *Pseudomonas syringae* pv. *tabaci* could induce transcripts for phenylalanine ammonia-lyase (PAL), chalcone synthase, chalcone isomerase, chitinase, and the production of phytoalexins in bean. Brown et al. (1995) have shown that strains of *Xanthomonas campestris* pv. *vesicatoria* with mutations in the *hrp* gene cluster induce the formation of large papillae and other cell wall changes in adjacent plant cells when inoculated into pepper. Amorphous material, which appears to encapsulate the *hrp* mutant bacteria, contains components that are cross-reactive immunologically with hydroxyproline-rich glycoproteins from tomato. In contrast, only minor cell wall alterations are observed in response to the cognate wild-type strain (a compatible interaction). These observations indicate that the formation of papillae is activated by the recognition of determinants carried by *X. campestris* pv. *vesicatoria* that do not require the *hrp* genes for their action. In addition, the observations imply that the *hrp*-encoded secretion system delivers effectors that suppress the induction of such responses.

The aim of our work here was to discover additional biochemical determinants in pepper that are potential targets for modulation by the action of *hrp* genes. We did this by studying aspects of phenolic metabolism in pepper leaves (*Capsicum annuum*) inoculated with strains of *X. campestris* and *hrp* mutant derivatives. *C. annuum* cv. Early Calwonder ECW 10R carries the *BsI* gene, which confers resistance against *X. campestris* pv. *vesicatoria* strains carrying the avirulence gene *avrBsI* (a gene-for-gene interaction). In addition, this pepper

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cultivar exhibits nonhost resistance to *X. campestris* pv. *campestris*, a pathogen of crucifers. We show that the nonhost resistance of pepper to *X. campestris* pv. *campestris* and the gene-for-gene-determined resistance of pepper to strains of *X. campestris* pv. *vesicatoria* is associated with the induction of the synthesis of hydroxycinnamic acid conjugates with tyramine, in particular feruloyl-tyramine (FT) and coumaroyl-tyramine (CT). The synthesis of these compounds previously has been associated with the resistance of plants to fungi and viruses (Keller et al. 1996; Negrel and Jeandet 1987).

Synthesis of FT and CT occurs by condensation of the CoA derivatives of coumaric acid and ferulic acid with tyramine, a reaction catalyzed by the enzyme tyramine hydroxycinnamoyl transferase (THT; EC 2.3.1.110). The CoA derivatives are synthesized via the phenylpropanoid pathway, the first committed step of which is catalyzed by the enzyme PAL (EC 4.3.1.5). Tyramine is derived from L-tyrosine by the action of tyrosine decarboxylase (TyDC; EC 4.1.1.25). This biosynthetic scheme is shown in Figure 1. We show that the synthesis of FT and CT in pepper induced by avirulent bacteria is associated with increased levels of transcripts for *PAL* and *THT* and an increase in TyDC activity. Analysis of effects induced by *hrp* mutants of the different strains revealed the existence of *hrp* gene-dependent and -independent mecha-

nisms for induction of FT and CT synthesis. The *hrp* gene-independent mechanism, which was found only in *X. campestris* pv. *campestris*, was characterized by a relatively rapid induction of TyDC activity and accelerated synthesis of FT and CT.

RESULTS

FT and CT synthesis is associated with gene-for-gene and nonhost resistance of pepper to *X. campestris* pathovars.

Inoculation of pepper leaves with either of the avirulent strains *X. campestris* pv. *vesicatoria/avrBs1* or *X. campestris* pv. *campestris* at 10^7 CFU/ml led to a HR, although with different timings. With *X. campestris* pv. *campestris*, visible HR occurred 20 to 24 h postinoculation (hpi), whereas with *X. campestris* pv. *vesicatoria/avrBs1*, HR was more rapid, occurring 12 to 16 hpi. In contrast, inoculation of pepper leaves with the virulent strain *X. campestris* pv. *vesicatoria* at 10^7 CFU/ml caused no macroscopic symptoms within 24 hpi, and disease symptoms developed only at later time points (3 days postinoculation). We analyzed the subset of phenolic compounds, which partition into ethyl acetate from an extract made initially from leaves with 90% methanol, as outlined below. This subset of compounds includes salicylic acid (SA), whose synthesis is associated with disease resistance in many plants (Dempsey et al. 1999). In initial experiments, extracts from leaves 24 hpi with *X. campestris* pv. *campestris* were compared with extracts from leaves inoculated with water by reverse-phase high-pressure liquid chromatography (HPLC) with diode array detection. The HPLC profiles at 306 nm showed a characteristic set of five peaks in the extracts from leaves inoculated with *X. campestris* pv. *campestris*. These peaks, however, were absent in extracts from uninoculated leaves (SA does not absorb at 306 nm and is detected at lower wavelengths) (Fig. 2A and B). The two major peaks (Fig. 2A, 2 and 4) were identified as *trans* CT and *trans* FT by the following criteria. The UV spectra of the compounds (Fig. 2C and D) were identical to that of authentic CT and FT, respectively. Secondly, the compounds cochromatographed with authentic standards. Finally, the values for $M + 1$ for the material in the two peaks were determined on the SELDI mass spectrometer to be 311 and 288, respectively, which is identical to authentic FT and CT. Peaks 3 and 5 in the chromatogram (Fig. 2A) were identified as the *cis* derivatives of FT and CT. Peak 1 was tentatively identified from its UV spectrum as a flavonoid, possibly an apigenin derivative, but this compound was not studied further in this work.

Analysis of the time course of induction of FT and CT synthesis in response to *X. campestris* pv. *campestris* is shown in Figure 3A and B. CT and FT accumulated 12 to 24 hpi in this incompatible (nonhost) interaction. In the gene-for-gene-determined incompatible response to *X. campestris* pv. *vesicatoria/avrBs1*, FT and CT synthesis was induced more rapidly (Fig. 3D and E). The relative timing of these responses reflected the relative timing of the HR collapse (see above). In contrast, only traces of CT and FT were detected in pepper tissue inoculated with the virulent bacterium *X. campestris* pv. *vesicatoria* (Fig. 3D and E) or in water-inoculated controls. Analysis of the levels of SA in the same extracts showed similar induction kinetics to FT and CT. In leaves inoculated with *X. campestris* pv. *campestris* (Fig. 3C), SA levels increased 12

Fig. 1. Biosynthetic pathway for coumaroyl-tyramine (CT) and feruloyl-tyramine (FT). CT and FT are synthesized by the condensation of tyramine derived from tyrosine by the action of tyrosine decarboxylase (TyDC) and coumaroyl- or feruloyl-CoA derived from the phenylpropanoid pathway. The first committed step of the phenylpropanoid pathway is catalyzed by the enzyme phenylalanine ammonia lyase (PAL). The condensation of tyramine and coumaroyl- or feruloyl-CoA is catalyzed by the enzyme tyramine hydroxycinnamoyl transferase (THT).

to 24 hpi. In leaves inoculated with *X. campestris* pv. *vesicatoria/avrBs1*, SA synthesis was initiated more rapidly 4 to 12 hpi (Fig. 3F). In contrast, SA did not accumulate in response to virulent *X. campestris* pv. *vesicatoria* (Fig. 3F) for up to 24 hpi or in water-inoculated controls.

FT and CT have antimicrobial activity against *X. campestris*.

Synthetic FT and CT were added to liquid cultures of *X. campestris* pv. *campestris* at various final concentrations, and the effect on bacterial growth was monitored over a 72-h period. At a final concentration of 250 μ M, FT and CT were bactericidal. No viable bacteria were left 24 h after the addition of FT, whereas all viable bacteria were eliminated 48 to 72 h after the addition of CT (Fig. 4). At 25 μ M, the effects of FT and CT were less pronounced and FT reduced the growth of *X. campestris* pv. *campestris* 15-fold compared to the control at 72 h, whereas CT reduced the growth of *X. campestris* pv. *campestris* approximately fivefold over the same time course. At 2.5 μ M, neither compound affected the growth of *X. campestris* pv. *campestris*.

FT and CT synthesis is associated with transcript accumulation for THT, PAL, and increased TyDC activity.

The synthesis of CT and FT could potentially be regulated by the supply of immediate biosynthetic precursors, tyramine, and CoA esters of hydroxycinnamic acids as well as through alterations in the levels of condensing enzyme THT (Fig. 1). The availability of gene probes for *PAL* and *THT* derived from other solanaceous plants allowed for analysis of the transcript levels of these genes in inoculated pepper leaves. Unfortunately, although genes or cDNAs for TyDC have been isolated from a number of nonsolanaceous plant sources, none of these were useful to detect TyDC transcripts in pepper (M.-A. Newman, unpublished data). Consequently, we were restricted to measurements of the extractable TyDC enzyme activity to estimate the effects of bacterial inoculation on the biosynthesis of tyramine. Inoculation of pepper leaves with *X. campestris* pv. *campestris* (Fig. 5) or with *X. campestris* pv. *vesicatoria/avrBs1* (data not shown) induced an increased transcription of *PAL* and *THT* genes. The accumulation of both transcripts in response to *X. campestris* pv. *campestris* was maximal at 12 hpi, and levels were considerably lower at 24 hpi (Fig. 5). Similar time courses and levels of induction were seen in response to *X. campestris* pv. *vesicatoria/avrBs1* (data not shown). In contrast, no transcripts for *PAL* or *THT* were detected in uninoculated leaves, control leaves infiltrated with water (Fig. 5) or in leaves inoculated with the virulent *X. campestris* pv. *vesicatoria*.

No TyDC enzyme activity could be detected in uninoculated plants. Inoculation with either *X. campestris* pv. *campestris* or with *X. campestris* pv. *vesicatoria/avrBs1* (both avirulent) caused a marked, although transient, increase in activity with a maximum at 12 hpi. Results for *X. campestris* pv. *campestris* are shown in Figure 6A. No change in TyDC activity occurred in response to infiltration with water or inoculation with the virulent *X. campestris* pv. *vesicatoria*. Overall, the results demonstrate that the accumulation of FT and CT, which occurred in response to inoculation with avirulent bacteria, was preceded by activation of the phenylpropanoid pathway and the biosynthetic pathway to tyramine as well as the final condensing step.

An alternative pathway for the formation of FT and CT would be their release by hydrolysis from conjugated storage forms, which for many phenolic compounds are glycosyl derivatives. The possible presence of glycosyl derivatives of FT and CT in untreated pepper leaves was assessed by the standard HPLC method after treatment of methanol extracts with almond β -glucosidase. This enzyme treatment did not release any free CT or FT, indicating the absence of preformed glycosylated derivatives.

The *hrp* deletion mutant of *X. campestris* pv. *campestris* induces a rapid synthesis of FT and CT.

The induction of the HR collapse in pepper by *X. campestris* pv. *campestris* and *X. campestris* pv. *vesicatoria/avrBs1* depends upon the presence of an intact *hrp* gene cluster in the bacteria. To investigate the relationship between the HR collapse and the accumulation of FT and CT, we studied the responses of pepper to mutant strains carrying deletions of all or part of the *hrp* gene cluster. *X. campestris* pv. *campestris* strain 8420, a mutant in which the full *hrp* gene cluster has been deleted (Liddle 1992), induced synthesis of FT and CT, which was much more rapid than that induced by the cognate wild type, occurring 2 to 4 hpi. (Fig. 7). The levels of FT and CT per gram of fresh weight of leaf were 0.30 and 0.52 μ g, respectively, at 4 hpi. These levels were maintained at 8, 12, and up to 24 hpi. In contrast to these effects in the nonhost interaction, *X. campestris* pv. *vesicatoria/avrBs1* mutants carrying deletions of the *hrpD* operon induced barely detectable levels of FT and CT (< 1 ng per g of fresh weight) at 24 hpi. Deletion of the *hrpD* operon in the virulent *X. campestris* pv. *vesicatoria* strain did not influence the kinetics of accumula-

Fig 2. Presence of hydroxycinnamoyl-tyramine conjugates in pepper leaves inoculated with *Xanthomonas campestris* pv. *campestris*. **A**, High-pressure liquid chromatography (HPLC) analysis (detection at 306 nm) of ethyl acetate-extracted pepper leaves 24 h postinoculation of *X. campestris* pv. *campestris* (10^7 CFU/ml of inoculum). The section of the chromatogram at 30–36 min is shown. Peaks: 1, unidentified flavonoid (apigenin derivative?); 2, *trans* coumaroyl-tyramine (CT); 3, *cis* CT; 4, *trans* feruloyl-tyramine (FT); 5, *cis* FT. **B**, HPLC analysis of ethyl acetate-extracted pepper leaves 24 h postinoculation with water. **C**, Absorbance spectrum of putative *trans* CT. **D**, Absorbance spectrum of putative *trans* FT, as recorded by diode array detection.

tion or levels of FT and CT, which remained very low. In contrast to the opposing effects on FT and CT synthesis, deletion of *hrp* genes in *X. campestris* pv. *campestris* and *X. campestris* pv. *vesicatoria*/avrBs1 eliminated the ability of these strains to induce SA synthesis in pepper (data not shown).

The *hrp* deletion mutant of *X. campestris* pv. *campestris* induces a rapid increase in TyDC activity in pepper.

In order to investigate the possible basis for the accelerated synthesis of FT and CT in response to the *hrp* mutant of *X. campestris* pv. *campestris*, the transcription of *PAL* and *THT* and TyDC activity were assessed as before. Deletion of the *hrp* gene cluster in *X. campestris* pv. *campestris* had no effects on the timing of accumulation of transcripts for *PAL* and *THT*, which were maximal at 12 hpi. Substantial effects were seen on the timing of the induction of TyDC activity, however, which occurred far more rapidly (within 1 hpi) in response to the *hrp* deletion mutant of *X. campestris* pv. *campestris* than in response to the wild type (Fig. 6B). The *X. campestris* pv. *vesicatoria* mutant carrying a deletion of the *hrpD* operon did not induce accumulation of *PAL* or *THT* transcripts or elicit any increase in TyDC activity (data not shown).

DISCUSSION

Conjugates of hydroxycinnamic acids with tyramine, derivatives of tyramine such as dopamine and methoxytyramine, or polyamines such as putrescine or agmatine are found in a wide range of plants, including barley, onion, potato, and tobacco (Keller et al. 1996; McLusky et al. 1999; Negrel and Martin 1984; Negrel and Jeandet 1987; Von Roepenack et al. 1998). It has been shown that their synthesis is activated in response to fungal elicitors, attempted infection by fungi and viruses, and, in some cases, wounding. Increased synthesis of FT and CT is associated with *Phytophthora infestans*-elicitor stimulation of potato cell suspension cultures (Schmidt et al. 1998) and the resistance of potato plants to *P. infestans* (Keller et al. 1996). Biosynthesis of FT is induced in tobacco by *Tobacco mosaic virus* infection (Negrel and Jeandet 1987). In tomato, potato, and tobacco, accumulation of FT occurs after wounding, although in wounded pepper and nightshade, no FT could be detected (Pearce et al. 1998). Here, we have shown that synthesis of FT and CT is associated with the nonhost- and gene-for-gene-determined resistance reactions of pepper to *X. campestris*. As far as we are aware, this is the first ex-

Fig. 3. Time course of accumulation of phenolics in pepper tissue in response to different strains of *Xanthomonas campestris*. *X. campestris* pv. *campestris* (*Xcc*), *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*), and *Xanthomonas campestris* pv. *vesicatoria* carrying the avirulence gene *avrBs1* (*Xcv/avrBs1*) were inoculated into pepper leaves at 10^7 CFU/ml. The levels of **A and D**, coumaroyl-tyramine; **B and E**, feruloyl-tyramine; and **C and F**, salicylic acid are the mean and standard deviation of three separate measurements at each time point. **A–C**, *Xcc*; **D–F**, *Xcv* and *Xcv/avrBs1*.

ample of these compounds being induced during a plant interaction with bacteria. In these resistance responses, the induction of FT and CT synthesis shows a similar time course to the induction of SA synthesis. Although the *trans* isomers of FT and CT predominated, the *cis* isomers of FT and CT also were recovered from infected plants. It was considered likely that these forms were generated artefactually from the *trans* forms during the extraction procedure.

Hydroxycinnamoyl-tyramine conjugates are suggested to have two roles in resistance. They can be incorporated into the plant cell wall to strengthen it against microbial degradation or potentially they can act directly as antimicrobial agents. Grandmaison et al. (1993) showed that FT induced hyphal branching but reduced total fungal growth of mycorrhizal fungi. Not all fungi are sensitive to the action of hydroxycinnamoyl tyramines, however, and McLusky et al. (1999) failed to detect any antifungal activity against *Botrytis* spp. We have found that soluble FT and CT have antibacterial activity against *X. campestris* pv. *campestris* and therefore may play a direct role in plant resistance to bacteria. On the basis of the data in Figure 3 and the assumption that the compounds are distributed throughout the cellular water, concentrations of FT and CT in plants 24 hpi with *X. campestris* pv. *campestris* can be calculated to 4 and 15 μ M, respectively. These levels are below those that give significant growth inhibition in vitro. Localization of FT and CT to the apoplastic space, however, would increase their effective concentration in the immediate bacterial environment. Such localization is plausible because these compounds are known to undergo incorporation into the cell wall. Such incorporation could account for the reduction in the levels of soluble FT seen at later time points in plants inoculated with *X. campestris* pv. *vesicatoria*/avrBs1. A definitive role for CT and FT in disease resistance in any plant, however, has yet to be established.

To study the effects of mutation of the *hrp* genes on plant responses, we used different mutants in the different *X. campestris* pathovars: a *hrpD* deletion mutant in *X. campestris*

pv. *vesicatoria* and a deletion of the full *hrp* gene cluster in *X. campestris* pv. *campestris*. The *hrpD* operon of *X. campestris* pv. *vesicatoria* comprises six genes, three of which (*hrcQ*, *hrcR*, and *hrcS*) are predicted to encode conserved components of a type III protein secretion system (Huguet et al. 1998). Hence, the deletions in both strains will lead to the loss of the Hrp secretion pathway. The induction of FT and CT synthesis by *X. campestris* pv. *vesicatoria* depends on the presence of *avrBs1* and a functional Hrp secretion pathway. In contrast, the results suggest the existence in *X. campestris* pv. *campestris* of a *hrp* gene-independent mechanism for the induction of hydroxycinnamoyl-tyramines. The increase in the synthesis of FT and CT induced by wild-type avirulent bacteria is associated with the activation of synthetic pathways for tyramine and for feruloyl- and coumaroyl-CoA and increased transcription of genes encoding THT. In contrast, the rapid (but restricted) synthesis of FT and CT in response to the *hrp* mutant of *X. campestris* pv. *campestris* is associated only with an accelerated activation of TyDC and does not appear to require alterations in the timing of induction of *PAL* and *THT* genes. In this case, FT and CT synthesis may depend upon preexisting THT enzyme and pools of feruloyl- and coumaroyl-CoA.

The more rapid induction of FT and CT by *hrp* mutants of *X. campestris* pv. *campestris* compared with the wild type also suggests the existence of an *hrp* gene-dependent mechanism to modulate the nonspecific induction of this defense-related response. Furthermore, the results identify TyDC activity or levels as a target for modulation. Exactly how this is achieved is not clear. One plausible mechanism is an effect on the plant cells through the suppression of TyDC induction by bacterial effectors delivered by the Hrp secretion system. Alternatively, the *hrp* genes could regulate processes in the bacteria that modify the levels or activities of the bacterial inducer(s) of TyDC.

An increase in TyDC activity is a common response of many plants to attempted infection by fungi and viruses and to treatment with fungal elicitors (Kawalleck et al. 1993; Keller et al. 1996; Negrel and Jeandet 1987; Trezzini et al. 1993). In the cases where this response has been studied, strong increases in activity have been shown to be the result of rapid gene activation. Transcriptional activation of *TyDC* genes in cell suspensions of parsley and *Arabidopsis thaliana* occurs 1 to 1.5 h after the addition of fungal elicitor (Kawalleck et al. 1993; Trezzini et al. 1993). The product of TyDC action can

Fig. 4. Antibacterial activity of coumaroyl-tyramine (CT) and feruloyl-tyramine (FT) against *Xanthomonas campestris* pv. *campestris*. Bacterial cultures in liquid minimal medium (MMXC) were supplemented with solutions of CT or FT in dimethylformamide to a final concentration of 250 μ M. Control cultures received dimethylformamide alone. The number of viable bacteria at each time point was assessed by serial dilution and plating. The values given are the mean and standard deviation of three separate measurements.

Fig. 5. Accumulation of transcripts for phenylalanine ammonia lyase (PAL) and tyramine hydroxycinnamoyl transferase (THT) in pepper in response to *Xanthomonas campestris* pv. *campestris*. Northern blot of 10 μ g of total RNA extracted from pepper leaves at different times postinoculation with *X. campestris* pv. *campestris* (10^7 CFU/ml start inoculum) was probed with **A**, *PAL* cDNA and **B**, *THT* cDNA. **C**, rRNA loading control. Lane 1: no treatment; lane 2–5: water infiltration at 0, 4, 12, and 24 h posttreatment; lanes 6–9: *X. campestris* pv. *campestris* inoculation at 0, 4, 12, and 24 h posttreatment.

be incorporated directly into the plant cell wall (Keller et al. 1996; Negrel and Jeandet 1987) as well as into hydroxycinnamoyl-tyramines. Alterations of the induction kinetics of TyDC by bacterial pathogens could promote pathogenesis by preventing or delaying the production of antibacterial compounds and reinforcement of the plant cell wall.

The existence of general nonspecific mechanisms of defense-gene activation and their suppression by wild-type bacteria has been suggested previously (Brown et al. 1995; Jakobek and Lindgren 1993; Jakobek et al. 1993), although the elicitors of such responses and their distribution among different bacteria remain largely undefined. Potential elicitors could include cell surface components such as lipopolysaccharides, cell surface polysaccharides such as the K-antigen (Becquart-de Kozak et al. 1997), bacterial flagellin (Felix et al. 1999), or extracellular proteins and enzymes that are not transported by the *hrp* gene-encoded type III systems (Davis et al. 1984; Palva et al. 1993; Vidal et al. 1997). There is little information on the range of plant responses induced by such elicitors. Our results are consistent with the view that *X. campestris* pv. *campestris* carries an elicitor of TyDC activity, which is absent in *X. campestris* pv. *vesicatoria*. Nevertheless, strains of *X. campestris* pv. *vesicatoria* do carry general elicitors of other plant defense responses because a range of *X. campestris* pv. *vesicatoria* strains with mutations in the *hrp* gene cluster are able to induce the formation of papillae in pepper (Brown et al. 1995).

Many nonhost interactions result in a HR and, in some cases, the *avr* genes in the pathogen that condition these effects in the nonhost have been identified (Fillingham et al. 1992; Kobayashi et al. 1989). Nevertheless, the induction of *hrp* gene-independent defense responses may determine the outcome of interactions between plant pathogenic bacteria and nonhosts in some cases. In *A. thaliana* wild-type *X. campestris* pv. *vesicatoria* does not induce HR, but the wild-type and a *hrpA* mutant of *X. campestris* pv. *vesicatoria* induce papilla formation (Brown et al. 1995). The triggering of plant defense mechanisms by *hrp*-independent inducers also could play a

Fig. 6. Tyrosine decarboxylase (TyDC) enzyme activity in pepper leaves after inoculation with **A**, wild-type *Xanthomonas campestris* pv. *campestris*. **B**, *hrp* deletion mutant of *X. campestris* pv. *campestris*. Bacteria were inoculated at 10^7 CFU/ml of start inoculum. The mean and standard deviation of three separate measurements at each time point are given. No TyDC activity was detected in untreated or water-treated tissue (data not shown).

role in the restriction of opportunistic pathogens and saprophytic bacteria.

MATERIALS AND METHODS

Bacterial strains, plant material, and plant challenge with pathogens.

The *X. campestris* pv. *campestris* strains used were strain 8004, a rifampicin-resistant mutant of a wild-type isolate of *X. campestris* pv. *campestris* (Daniels et al. 1984) and strain 8420, a *hrp* deletion mutant of strain 8004 (Liddle 1992). The *X. campestris* pv. *vesicatoria* strains used were wild-type *X. campestris* pv. *vesicatoria* strain 82.8 (Stall et al. 1986), the near-isogenic strain 82.8 carrying the avirulence gene *avrBs1* on a plasmid (Ronald and Staskawicz 1988), a *hrpD* deletion mutant of strain 82.8 (82.8 *hrpD*; a gift from U. Bonas, Halle, Germany), and strain 82.8 *hrpD*-*avrBs1* (this study). All strains were cultured normally in peptone-yeast extract-glycerol medium at 28°C, as described by Turner et al. (1984).

The plant material used for all experiments was Early Calwonder ECW 10R, which carries the resistance gene *Bs1*. This gene conditions plant resistance to strains of *X. campestris* pv. *vesicatoria*, the casual agent for bacterial spot disease, that carry the avirulence gene *avrBs1* (a gene-for-gene interaction). Strains without *avrBs1* are virulent on Early Calwonder ECW 10R. Pepper is a nonhost for *X. campestris* pv. *campestris*, which is a pathogen of brassicas. Growth conditions for plants were described previously (Newman et al. 1994). Bacterial suspensions in water at a concentration of 10^7 CFU/ml were

Fig. 7. High-pressure liquid chromatography traces (detection at 306 nm) of ethyl acetate-extracted pepper leaf tissue 4 h posttreatment with strains of *Xanthomonas campestris* pv. *campestris*. Traces are **A**, *X. campestris* pv. *campestris* *hrp* deletion mutant strain 8420; **B**, *X. campestris* pv. *campestris* wild-type strain 8004; and **C**, water-treated control. Profiles for the *hrp* deletion mutant at 8, 12, and 24 h postinoculation were similar to those at 4 h postinoculation (data not shown).

infiltrated into mature leaves of plants, as described by Collinge et al. (1987). Control leaves were inoculated with water. The inoculated plants were placed in a growth cabinet at 25°C with 16 h of light.

Chemical synthesis of FT and CT.

FT and CT were synthesized chemically, following the method described by Villegas and Brodelius (1990). The purity and identity of the phenolics were confirmed by comparison of UV absorbance spectra and by cochromatography in thin-layer chromatography and HPLC, with standards authenticated by HPLC-electrospray mass spectrometry (a gift from M. Bennett, Wye College, U.K.). The molecular weight and purity of the synthesized compounds also was checked with the SELDI mass spectrometer (Ciphergen Biosystems, Fremont, CA, U.S.A.).

Determination of FT, CT, and SA levels.

The procedure for FT, CT, and SA extraction and determination was adapted from the method described by Raskin et al. (1989). Phenolic compounds were extracted from plant tissue with 90% methanol followed by ethyl acetate extraction. To assess the presence of glycosylated derivatives of FT and CT in leaves, methanol extracts were dried, dissolved in 0.1 M sodium acetate buffer (pH 5.2), and treated with 80 units of almond β -glucosidase (Sigma, Poole, U.K.) per ml for 90 min before ethyl acetate extraction. HPLC separation was performed with a Columbus 5- μ m C₁₈ reverse-phase column (25 cm $\times$ 4.6 mm; Phenomenex, Macclesfield, U.K.). The following binary gradient, with a flow rate of 1 ml per min, was used for elution: $T = 0$ 90% solvent A (1 mM trifluoroacetic acid), 10% solvent B (100% acetonitrile); $T = 50$ min, 60% solvent A, 40% solvent B; $T = 60$ min, 90% solvent A, 10% solvent B. SA concentrations were calculated from absorbencies at 200 and 235 nm and FT and CT concentrations at 270 and 306 nm. Absorbencies were measured with a diode array scanning UV detector (Gilson, Middleton, WI, U.S.A.). Identities of the phenolics were confirmed by cochromatography and mass spectrometry (see above).

Antimicrobial activity of FT and CT.

X. campestris pv. *campestris* cultures grown overnight at 28°C in a rotary shaker in MMXC minimal media (Barber et al. 1997) were adjusted to a concentration of 5×10^5 CFU/ml. Synthetic CT or FT, dissolved in dimethylformamide (DMF), were added to these cultures to provide a range of final concentrations. Control cultures received an equal volume of DMF alone. The cultures were returned to the rotary shaker, and the viable counts of bacteria were measured over 72 h by plating serial dilutions onto plates. The experiment was performed on triplicate cultures and repeated three times. At the levels used, DMF had no effect on bacterial growth.

RNA isolation and characterization.

Total RNA was extracted from frozen leaf material at various time points after inoculation of *X. campestris* strains and from control tissue with use of the guanidine hydrochloride method described by Logemann et al. (1987). For Northern blots, RNA samples (10 μ g of total RNA) were separated on formaldehyde-agarose gels and transferred to Hybond-N nylon membranes (Nycomed Amersham, Little Chalfont, Buck-

inghamshire, U.K.), according to the manufacturer's protocol. The integrity of the RNA was assessed by visualization of ribosomal RNA by ethidium bromide staining. The cDNA probes (Lee et al. 1992 [PAL]; Schmidt et al. 1999 [THT]) were labeled with α -³²P-dCTP with a random priming kit (Nycomed Amersham). RNA gel blots were hybridized and washed, as described in Newman et al. (1994). The hybridized blots were exposed on a Fuji Imaging Plate and analyzed on a FUJIX BAS 100 bio-imaging analyzer (Fuji Photo Film, Tokyo, Japan).

Protein extract preparation and TyDC enzyme assay.

Crude protein extraction from pepper leaf tissue and subsequent TyDC enzyme assay was performed exactly in accordance with the method described by Schmidt et al. (1998). The product (tyramine) formation was determined by HPLC using a Hypersil 5- μ m C₈ reverse-phase column (25 cm $\times$ 4.6 mm; Phenomenex). The following binary gradient, with a flow rate of 1 ml per min, was used for elution: $T = 0$ min, 100% solvent A (0.1 M ammonium acetate/citrate [pH 6.5]), 0% solvent B (80% aqueous acetonitrile); $T = 25$ min, 65% solvent A, 35% solvent B; $T = 35$ min, 30% solvent A, 70% solvent B; $T = 40$ min, 5% solvent A, 95% solvent B; $T = 45$ min, 100% solvent A, 0% solvent B. Quantification of tyramine produced by the TyDC activity was achieved with a tyramine standard from Sigma. The absorbency of tyramine ($\lambda_{\max}$ at 275 nm) was measured with a Gilson diode array scanning UV detector. The experiment was repeated three times.

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