# Induction of Plant Defense Response by Exoenzymes of *Erwinia carotovora* subsp. *carotovora*

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***Erwinia carotovora* subsp. *carotovora*, the etiological agent of bacterial soft rot, produces a variety of plant cell wall-degrading exoenzymes that are the main virulence factors of this pathogen. To determine the role of these enzymes in the plant-bacterium interaction, individual exoenzymes were produced in *Escherichia coli* harboring cloned exoenzyme encoding genes from *E. c.* subsp. *carotovora* and applied to tobacco plants. The plant response was monitored by following the expression of a plant gene encoding a pathogenesis-related β-1,3-glucanase. The transcript for β-1,3-glucanase was shown to rapidly accumulate in plants treated with pectic enzymes, but not with a cellulase from *E. c.* subsp. *carotovora*. Both pectate lyase (Pel) and polygalacturonase (Peh) were shown to increase the host β-1,3-glucanase mRNA levels. In addition, a similar plant response could be elicited by the application of polygalacturonase-treated polypectate. *In planta* analysis of tobacco seedlings inoculated with reduced virulence mutants of the pathogen that still produced pectic enzymes resulted in accumulation of β-1,3-glucanase mRNA. However, no accumulation of β-1,3-glucanase mRNA was observed in plants inoculated with exoenzyme-negative mutants. These results indicate that pectic enzymes of *E. c.* subsp. *carotovora* probably elicit the plant defense response by releasing pectic fragments from the plant cell wall that may function as endogenous elicitors. Interestingly, infection of plants by the wild-type pathogen induced the plant response only weakly and transiently, suggesting that the wild-type bacteria are able to suppress the plant response. Induction of the plant defense by exoenzyme treatment conferred increased resistance against subsequent infections by *E. c.* subsp. *carotovora*.**

*Additional keyword:* oligosaccharide elicitior.

*Erwinia carotovora* subsp. *carotovora* is a bacterial soft-rot pathogen that is destructive to a wide range of plants both in culture and in storage (Pérombelon and Kellman 1980). The major symptom of the soft-rot disease is the maceration of the plant tissue caused by enzymes secreted by *E. c.* subsp. *carotovora*. These extracellular enzymes, including pectinases, cellulases, and proteases, that can degrade components of the plant cell wall are thought to be main determinants of pathogenicity in soft-rot erwinias (Collmer and Keen 1986; Kotoujansky 1987; Pirhonen *et al.* 1991). The pectic enzymes characterized from *E. c.* subsp. *carotovora* include several isoforms of endopectate lyase (Pel) (Hinton *et al.* 1989), an endopolygalacturonase (PehA) (Saarilahti *et al.* 1990a; Hinton *et al.* 1990), and a pectin lyase (Chatterjee *et al.* 1991). Two lines of evidence demonstrate the importance of the exoenzymes, particularly pectinases, in pathogenicity: First, bacterial mutants unable to produce or secrete the exoenzymes are avirulent (Andro *et al.* 1984; Pirhonen *et al.* 1991; Murata *et al.* 1991). Second, the soft-rot symptoms can be reproduced by acellular enzyme preparations from culture filtrates (Collmer and Keen 1986) or even in some cases by *Escherichia coli* strains harboring clones coding for pectinases from erwinias (Collmer and Keen 1986). In addition, marker exchange studies with a related soft-rot erwinia species, *Erwinia chrysanthemi*, have demonstrated that mutants lacking certain isoforms of pectate lyase (Pel) exhibit reduced virulence (Ried and Collmer 1988). Similar reduction of virulence was recently observed in PehA deficient mutants of *E. c.* subsp. *carotovora* (Saarilahti *et al.* 1992). Although a wealth of knowledge is available about the pectic enzyme genes and their regulatory properties *in vitro* (Collmer and Keen 1986; Kotoujansky 1987), only a limited number of *in planta* studies have been performed that increase our understanding of *Erwinia* pathogenesis and the plant response to *Erwinia* infection. Only recently have *in planta* experimental systems using potato tubers been presented for such studies on *Erwinia*-plant interaction (Yang *et al.* 1989, 1991, 1992; Rumeau *et al.* 1990).

Plants possess a large array of defenses, including several inducible systems that can be activated by pathogenic microorganisms, either by elicitor molecules from the pathogen or by plant cell wall components (endogenous elicitors) released by the action of the pathogen (Hahlbrock and Scheel 1987; Davis and Hahlbrock 1987; Collinge and Slusarenko 1987; Farmer *et al.* 1991). The pectic enzymes produced by a pathogen like *Erwinia* appear to play a role in this induction of plant defense reactions. Culture filtrates from *E. c.* subsp. *carotovora* or pectinase containing preparations from other organisms have been used to elicit, e.g., phytoalexin production (Davis *et al.* 1984; Hahlbrock and Scheel 1987) and recent work by Yang

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*et al.* (1992) indicates that endopectate lyase can release oligogalacturonides which are active as inducers of the plant phenylalanine ammonia-lyase (PAL). One of the best-characterized plant responses to pathogens, the induction of pathogenesis-related (PR) proteins (van Loon 1985; White and Antoniw 1991), is a rather universal response involved in many plant-pathogen interactions (Collinge and Slusarenko 1987), including that with *Erwinia* (Davis and Ausubel 1989; Castresana *et al.* 1990). Several PR proteins have been identified as β-1,3-glucanases (Kauffmann *et al.* 1987, 1990). The presence of both acidic (secreted) and basic (vacuolar) isoforms and their differential induction in response to external signals has been reported (Kauffmann *et al.* 1990; Memelink *et al.* 1990; Ward *et al.* 1991). There is evidence that β-1,3-glucanases can be induced in response to *Erwinia* infection (Castresana *et al.* 1990) or by treatment of plant cell cultures with *Erwinia* culture filtrates (Davis and Ausubel 1989).

In this report, we show that tobacco β-1,3-glucanase can be induced in response to *E. c.* subsp. *carotovora* infection and that this defense gene activation is caused by the action of the pectic enzymes produced by the pathogen.

## RESULTS

### Accumulation of β-1,3-glucanase mRNA is induced by pectic enzymes of *E. c.* subsp. *carotovora*.

Previous studies have demonstrated that pathogenesis-related proteins such as β-1,3-glucanases can be induced in plants by treatment with biotic elicitors, including pectin-degrading enzymes from various sources (Davis and Ausubel 1989; White and Antoniw 1991). To determine the response of plants to *E. c.* subsp. *carotovora* exoenzymes, acellular enzyme preparations (culture filtrates) were sprayed on *in vitro*-grown tobacco seedlings, and total RNA was isolated after times indicated. This RNA was subjected to Northern blot analysis using a cDNA corresponding to *gn1* β-1,3-glucanase gene from tobacco as a probe. Figure 1A shows that the β-1,3-glucanase mRNA accumulated rapidly in plants treated with the culture filtrate from the wild-type *E. c.* subsp. *carotovora* strain SCC3193. Maximum induction (more than 30-fold) was observed at 8 hr after elicitor treatment followed by a slow decline of the mRNA down to control levels 3–4 days after the treatment. In contrast, no accumulation of the mRNA could be observed in plants treated with culture filtrates from the exoenzyme-deficient mutant ($Exp^-$) SCC3065 (Fig. 1A). This suggests that the accumulation of the β-1,3-glucanase mRNA was indeed caused by the exoenzymes present in *E. c.* subsp. *carotovora* culture filtrates.

To confirm that the observed plant response was due to specific exoenzymes secreted by the pathogen and to analyze which of the exoenzymes produced by *E. c.* subsp. *carotovora* elicit the plant response, cloned exoenzyme encoding genes were introduced to *E. coli* and the enzymes prepared from individual *E. coli* clones. These enzymes that are normally secreted to the culture medium from *E. c.* subsp. *carotovora* are retained in the periplasm of *E. coli* (Saarilahti *et al.* 1990a, 1990b). Therefore, the enzymes produced in *E. coli* were first released by osmotic shock, and these preparations used for treatment of plants. The endopolygalacturonase (PehA, Saarilahti *et al.* 1990a) encoded by SCC3193 appeared to be a strong inducer of the plant β-1,3-glucanase mRNA accumulation (Fig. 1B). A strong plant response was also obtained with pectate lyase (Pel) containing preparations as exemplified by PelA (Fig. 1B). All four major endopectate lyases produced by SCC3193 (PelA, B, C, and D) gave a similar response (not shown). The kinetics of the β-1,3-glucanase mRNA accumulation was, however, markedly different with PehA

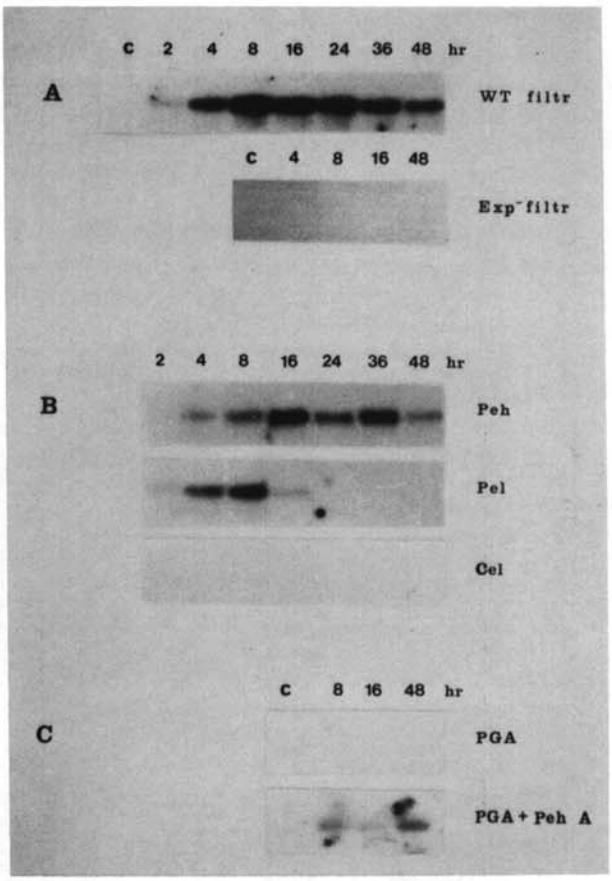


**Fig. 1.** Accumulation of β-1,3-glucanase mRNA in tobacco plants treated with *Erwinia carotovora* subsp. *carotovora* exoenzymes. Axenic seedlings of *Nicotiana tabacum* 'Samsun' were treated by spraying **A,** with acellular culture filtrates of *E. c.* subsp. *carotovora*; **B,** with periplasmic preparations of *Escherichia coli* harboring exoenzyme encoding plasmid clones; or **C,** with PGA preparations. Plant tissues were harvested at times indicated after treatment, the total RNA was isolated and 10-μg samples were subjected to Northern blot analysis. The RNA was blotted to nitrocellulose filters and hybridized to $^{32}$P-labeled tobacco *gn1* β-1,3-glucanase cDNA. **A,** The culture filtrates used came from *E. c.* subsp. *carotovora* wild-type strain SCC3193 (WT filtr) or the exoenzyme deficient $Exp^-$ strain SCC3065 ($Exp^-$ filtr) grown to stationary phase in L PGA medium. **B,** The periplasmic preparations used came from *E. coli* HB101 derivatives harboring cloned exoenzyme encoding genes from *E. c.* subsp. *carotovora* SCC3193 in pUC18 vectors. The genes *pehA*, *pelA*, and *celS* encode an endopolygalacturonase (Peh; Saarilahti *et al.* 1990a), an endopectate lyase (Pel; unpublished results from our laboratory), and a cellulase (CelS; Saarilahti *et al.* 1990b), respectively. **C,** Commercial sodium polypectate (PGA, Sigma P-1879) was incubated with a PehA preparation for 30 min at 37° C, the PehA inactivated by autoclaving before spraying on plants. The PGA control was treated similarly but without addition of PehA.

as compared to that obtained with the different Pels. Treatment by Pels resulted in a very rapid accumulation of the β-1,3-glucanase mRNA, but the amount of mRNA also decreased rapidly. Accumulation of the mRNA by PehA treatment was slower reaching a maximum, at 16 hr as compared to 8 hr with Pel, but persisted longer, up to 48 hr. This is comparable to the induction observed in plants treated with the *E. c.* subsp. *carotovora* culture filtrate (Fig. 1A). In contrast to the strong accumulation of the PR β-1,3-glucanase mRNA by the different pectic enzymes, no accumulation of this mRNA could be observed when plants were treated with cellulase (CelS, Saarilahti *et al.* 1990b). These results imply that the major pectate-degrading enzymes of *E. c.* subsp. *carotovora* are also the main elicitors of the plant response as monitored by following the expression of a defense-related β-1,3-glucanase gene. The most likely explanation for the observed response, enhanced β-1,3-glucanase gene expression, is that the pectic fragments released by the action of these enzymes function as endogenous elicitors of this and other defense-related genes. To test whether this is the case, commercial preparations of sodium polypectate (PGA, Sigma P-1879) was treated with PehA, and the partially hydrolyzed pectate was used to spray tobacco seedlings. The results of this analysis show (Fig. 1C) that PehA-treated PGA was indeed active as an elicitor of β-1,3-glucanase mRNA accumulation. The untreated preparations of PGA did not show β-1,3-glucanase inducing activity (Fig. 1C), neither did preparations of PGA treated with inactivated PehA.

**Growth of *E. c.* subsp. *carotovora* reduced virulent and avirulent mutants on tobacco.**

We have previously isolated a set of transposon-induced mutants of *E. c.* subsp. *carotovora* exhibiting either reduced virulent or avirulent phenotypes (Pirhonen *et al.* 1991). Here we employed two types of such mutants for analysis of the plant response: reduced virulent nonmotile mutants (Mot$^-$) that tend to produce small, slowly spreading lesions and avirulent mutants (Exp$^-$) affected in exoen-

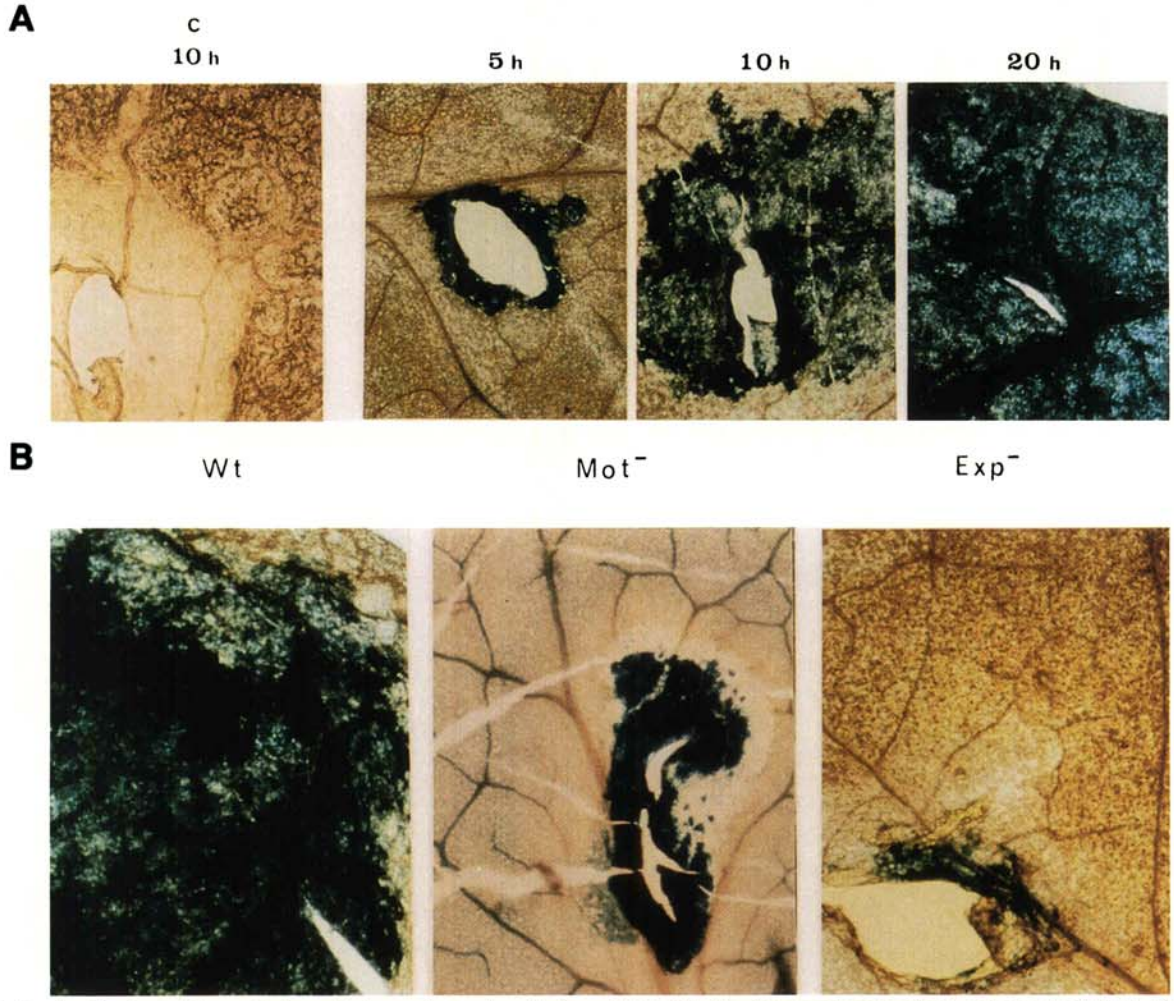


**Fig. 2.** *In situ* β-galactosidase staining of tobacco leaves locally inoculated with *Erwinia carotovora* subsp. *carotovora* strains. Single leaves of axenically grown *Nicotiana tabacum* 'Samsun' seedlings were punctured by a needle and $10^5$ cfu of bacteria resuspended into 10 mM $MgSO_4$ was applied. The inoculated leaves were harvested and stained for β-galactosidase. **A,** Comparison of plants inoculated with the control strain (C) SCC3193 (LacZ$^-$) and SCC3193 harboring the *ompR-lacZ* gene fusion plasmid pTK76 (LacZ$^+$). The leaves were harvested at times indicated after inoculation and stained for β-galactosidase. **B,** Comparison of infection of tobacco seedlings by the wild-type strain SCC3193 (WT), the reduced virulent nonmotile mutant SCC3111 (Mot$^-$), and the avirulent exoenzyme-deficient mutant SCC3065 (Exp$^-$). All strains harbored the *ompR-lacZ* fusion carrying plasmid pTK76.

zyme production. The Exp$^-$ mutants are virtually negative for all exoenzymes tested, in contrast to the Mot$^-$ strains that produce and secrete wild-type levels of these enzymes (Pirhonen *et al.* 1991).

Figure 2 shows the symptom development during wild-type *E. c.* subsp. *carotovora* infection. To be able to monitor the spreading of the bacteria *in planta* more easily we introduced a constitutively expressed *lacZ* gene from *E. coli* to the *E. c.* subsp. *carotovora* strains and developed an *in situ*-staining procedure for β-galactosidase. As suggested by Figure 2A, the wild-type bacteria (SCC3193) are spreading, along with the zone of maceration of the plant tissue caused by the exoenzymes. However, a different situation is seen with the mutants (Fig. 2B). The avirulent exoenzyme-deficient Exp$^-$ mutant (SCC3065) does not appear to multiply at all *in planta* (Fig. 3) and does not seem to cause any degradation of the plant tissue. The nonmotile Mot$^-$ mutant (SCC3111) produces wild-type levels of exoenzymes (Pirhonen *et al.* 1991), seems to multiply *in planta* after an initial lag similar to the wild-type strain, and causes maceration around the inoculation site (Figs. 2B and 3). In this case the zone of maceration appears to be spreading ahead of the bacteria (Fig. 2B). This is probably due to rapid diffusion of the secreted plant cell wall-degrading enzymes, while the spreading of the nonmotile bacteria producing them is slower. These results suggest that the living plant cells do not necessarily come in contact with the pathogen at all, but only with the enzymes secreted by the bacteria.

**Accumulation of β-1,3-glucanase mRNA can be induced during *E. c.* subsp. *carotovora* infection.**

To determine the effect of the wild-type and mutant *E. c.* subsp. *carotovora* infections on the plant response, seedlings were sprayed with bacterial cells, and the accumulation of the β-1,3-glucanase mRNA was monitored in the infected plantlets (Fig. 4). Plants did not appear to react to the *E. coli* control nor did we observe any β-1,3-glucanase mRNA accumulation in plants treated with the exoenzyme-deficient Exp$^-$ mutant. In contrast, plants responded strongly to the nonmotile exoenzyme producing strain SCC3111 by rapid and persistent accumulation of the β-1,3-glucanase transcript. This is in agreement with the results showing β-1,3-glucanase induction by acellular enzyme preparations (Fig. 1) and confirms the role of these enzymes in eliciting the plant response.

The plant response to the wild-type *E. c.* subsp. *carotovora* exhibited a different pattern as compared to the nonmotile mutant or to the effect of culture filtrates. We could observe accumulation of β-1,3-glucanase transcript (Fig. 4), but the induction was rather weak and transient. As the infection proceeded the amount of β-1,3-glucanase mRNA rapidly decreased. This would imply that the wild-type strain is capable of suppressing the plant response.

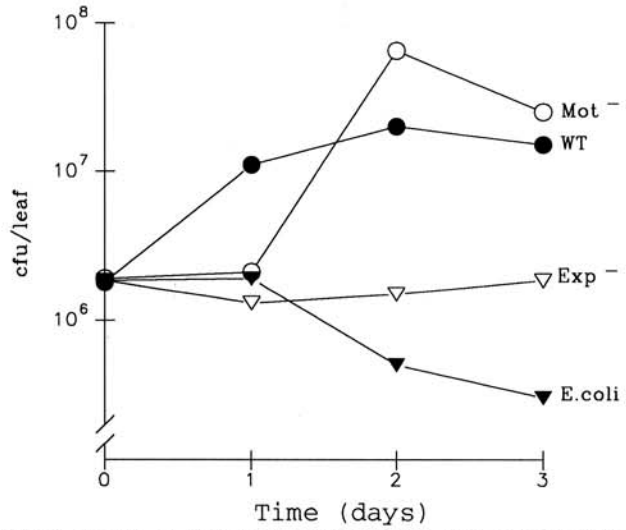


**Fig. 3.** Growth of *Erwinia carotovora* subsp. *carotovora* wild-type and mutant strains *in planta*. Tobacco seedlings were locally inoculated with overnight grown cultures of the bacteria at $10^6$ cfu/inoculum. At times indicated, the inoculated leaves were harvested and the amount of bacteria in each leaf determined by plating serial dilutions of leaf extracts onto L medium. Each point represents the mean of 10 individually inoculated leaves. The *E. c.* subsp. *carotovora* strains used were SCC3193, wild-type (WT, ●); SCC3111, a nonmotile mutant (Mot$^-$, ○) and SCC3065, an exoenzyme-deficient mutant (Exp$^-$, ▽). The *Escherichia coli* strain MC4100 (E. coli, ▼) was used as a control.

**Treatment of plants with exoenzymes induce resistance against subsequent *E. c.* subsp. *carotovora* infection.**

Induction of plant defense response by exogenous application of the signal compound, salicylic acid, has been

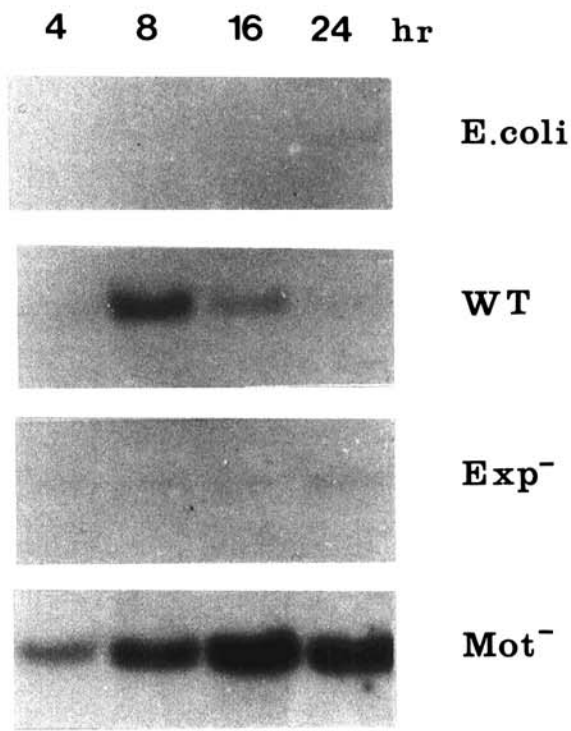


**Fig. 4.** Accumulation of β-1,3-glucanase mRNA in tobacco seedlings inoculated with *Erwinia carotovora* subsp. *carotovora* strains. Axenic seedlings of *Nicotiana tabacum* 'Samsun' were inoculated by spraying bacterial dilutions at $10^8$ cfu/ml in 10 mM $MgSO_4$. The plant samples were harvested at times indicated after inoculation and the total RNA was isolated. Ten-microgram aliquots of the RNA samples were subjected to Northern blot analysis, as described in the caption to Figure 1. The bacterial strains used for inoculation were: *Escherichia coli* strain MC4100 (*E. coli*), and *E. c.* subsp. *carotovora* strains SCC3193 (WT), SCC3065 (Exp$^-$) and SCC3111 (Mot$^-$).

shown to result in increased tolerance to viral infections (White and Antoniw 1991). As we have shown (Fig. 1), acellular pectic enzyme preparations are strong inducers of plant defense as monitored by following the accumulation of mRNA corresponding to one class of PR proteins, β-1,3-glucanase. To determine whether this had any effect on plant resistance towards *E. c.* subsp. *carotovora*, plants were treated by these enzymes for 2 days and subsequently inoculated with the wild-type *E. c.* subsp. *carotovora* strains. The results of this analysis (Table 1) indicate that exoenzyme treatment indeed enhances the resistance of plants to *E. c.* subsp. *carotovora*. The protection observed was most prominent with culture filtrates of wild-type strains but almost as good protection could be observed in PehA treated plants. Pel treatment did also appear to protect plants but not as well as that with PehA or culture filtrate. The extent of protection correlated well with the observed pattern of β-1,3-glucanase mRNA accumulation by these enzyme preparations (c.f. Fig. 1).

## DISCUSSION

We have developed an *in planta* system using axenic tobacco seedlings (Pirhonen *et al.* 1991) for studies on *Erwinia*-plant interaction. Here we have demonstrated that such plants can be used to study the plant response to exoenzyme elicitors of *E. c.* subsp. *carotovora*. Using this system we could show that treatment of plants with culture filtrates of a wild-type *E. c.* subsp. *carotovora* strain but not of exoenzyme-deficient mutants resulted in rapid and prominent (30-fold) accumulation of mRNA related to β-1,3-glucanase. In accordance with our results, induction of β-1,3-glucanase mRNA has been observed in cell cultures of *A. thaliana* treated with culture filtrates of *E. c.* subsp. *carotovora* (Davis and Ausubel 1989).

It has been suggested that the elicitor activity in *Erwinia* culture filtrates is due to *endo*-Pel (Davis *et al.* 1984; Yang *et al.* 1992). We could dissect the plant β-1,3-glucanase-inducing activity of *E. c.* subsp. *carotovora* by cloning the relevant exoenzyme genes of the pathogen to *E. coli* and producing the enzymes in this bacterium. By treatment of the tobacco seedlings with acellular extracts from *E. coli* strains harboring individual exoenzyme clones, we could demonstrate that the four major *endo*-Pels as well as *endo*-Peh (PehA) were efficient elicitors of the plant response. The elicitor activity appeared specific to pectic enzymes as a cellulase (CelS) was completely inactive. The kinetics of β-1,3-glucanase mRNA accumulation by the two groups of pectic enzymes (Pel and Peh) was markedly different and could reflect their different mode of cleavage of the pectate molecules (Collmer and Keen 1986). However, taken together, the induction patterns obtained by the separate exoenzymes correspond well to that by crude culture filtrates of *E. c.* subsp. *carotovora*, suggesting that the main elicitor activity in these filtrates is due to the pectic enzymes. These enzymes most probably release pectic fragments from the plant cell walls that in turn function as endogenous elicitors of the plant response (Davis *et al.* 1984; Davis and Hahlbrock 1987; Yang *et al.* 1992). In accordance with this hypothesis we did observe accumulation of β-1,3-glucanase related mRNA when plants were sprayed with sodium polypectate that had been treated with endopolygalacturonase (PehA).

**Table 1.** Exoenzyme-induced protection of tobacco against *Erwinia carotovora* subsp. *carotovora* infection

| | Disease symptom development (days)[a] | | | | |
|---|---|---|---|---|---|
| **Pretreatment** | **1** | **2** | **3** | **4** | **5** |
| | (+) | + | ++ | +++ | +++ |
| CelS | (+) | + | ++ | +++ | +++ |
| PelA | − | (+) | ++ | +++ | +++ |
| PehA | − | (−) | (+) | + | + |
| Wild-type filtrate | − | (−) | (+) | + | + |

[a] The pathogenicity test was done as described previously using axenic *Nicotiana tabacum* seedlings grown in 24-well tissue culture plates (Pirhonen *et al.* 1991). The pretreatment of plantlets with acellular enzyme preparations was done as described in the legend to Figure 1. After 2 days the plants were inoculated by *E.c.* subsp. *carotovora* strain SCC3193 as described by Pirhonen *et al.* (1991). The symptom development was followed daily for 5 days and scored using a scale from − (no maceration of plants) to +++ (plant material in the well is totally macerated).

*In situ* staining of infected plants for bacterial β-galactosidase was employed to monitor the spreading of bacteria during infection. Using reduced virulent mutants of *E. c.* subsp. *carotovora*, we could demonstrate that the plants respond to exoenzyme producing strains of the pathogen by induction of β-1,3-glucanase gene expression. The results with nonmotile (Mot$^-$) mutants (Fig. 3) suggest that the plant cells may actually first come in contact with the exoenzymes produced by the pathogen and not with the bacteria themselves. However, when plants were inoculated with a wild-type *E. c.* subsp. *carotovora*, which can totally macerate the plantlets in a couple of days, only a weak and transient response of the plant was observed. This kind of delayed and weak host response is typical in compatible interactions between a pathogen and its host (Collinge and Slusarenko 1987; Hahlbrock and Scheel 1987). It is also in agreement with the results of Castresana *et al.* (1990), who observed only a threefold induction of the *gnl* gene by *E. c.* subsp. *carotovora* inoculation. Taken together the results suggest that the wild-type pathogen is capable of suppressing the plant response caused by the action of its own exoenzymes. The mechanism of this suppression is not clear but could involve a further breakdown of the pectic fragments destroying their elicitor activity. Such degradation of the pectic oligosaccharides could be caused by exoacting-pectic enzymes (Yang *et al.* 1992).

Treatment of plants with pectic enzymes (*endo*-Pel and *endo*-Peh) of *E. c.* subsp. *carotovora* resulted in a rapid and prominent induction of the plant response as monitored by accumulation of β-1,3-glucanase-related mRNA. This response was much more prominent than that observed when plants were infected with the wild-type pathogen. Consequently, the treatment of plants with acellular enzyme preparations could mimick an incompatible plant-pathogen interaction and could, therefore, result in resistance response. This was tested by subsequent inoculation of the treated plants with the wild-type pathogen, and we could show that the treated plants indeed exhibited enhanced resistance towards *E. c.* subsp. *carotovora*.

## MATERIALS AND METHODS

**Plant material and inoculation of plants.**

Axenic tobacco seedlings (*Nicotiana tabacum* 'Samsun') grown in MS-2 medium (Murashige and Skoog 1962) in 24-well tissue culture plates were used in all experiments (Pirhonen *et al.* 1991). The plants were grown at 25° C in controlled environment in 16-hr light regime (2 $W/m^2$) for 4–5 wk before inoculation or enzyme treatment. Local inoculation was by pipetting 0.1 $\mu$l of bacterial suspensions in late logarithmic phase ($10^9$ colony-forming units [cfu]/ml) to leaves punctured by an injection needle. Surface inoculations were by spraying bacterial suspensions diluted in 10 mM $MgSO_4$ onto plants (2 ml/plate). Treatment by acellular enzyme preparations was by similarly spraying the seedlings with enzymes adjusted according to their relative activities in *E. c.* subsp. *carotovora* culture supernatant (cf. Pirhonen *et al.* 1991). The amount of individual enzymes applied per tissue culture plate were as follows: Peh, 5 units; Pel, 0.1 units (approximately 25% of the activity in culture supernatant containing 4 Pel isoforms); and Cel, 0.3 units. The assay conditions and definitions of units were as previously (Pirhonen *et al.* 1991). The inoculated plants were incubated at 25° C 16-hr light under 100% humidity for times indicated.

**β-Galactosidase staining and light microscopy.**

*In situ* staining of infected plant tissue was modified from our previous β-galactosidase staining method described by Teeri *et al.* (1989). The leaves, carefully wrapped in Miracloth (Calbiochem, La Jolla, CA), were placed between two porous plastic supports and fixed in 4% glutaraldehyde for 3 hr at +4° C. To aid the penetration of the fix, after 1 hr the material was flash freezed three times in liquid nitrogen. The staining with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and postfixing were as described (Teeri *et al.* 1989); staining times were from 1–4 hr. The fixed and X-Gal-stained samples were mounted with Entellan (Merck, Germany) and photographed with Olympus IMT2 microscope using Kodak Ektachrome 50 film.

**Bacteria and exoenzyme preparations.**

The wild-type *E. c.* subsp. *carotovora* strain used, SCC3193, has been described previously (Pirhonen *et al.* 1988). The transposon insertion mutants SCC3065 ($Exp^-$), defective in exoenzyme production, and the nonmotile mutant SCC3111 ($Mot^-$) have been characterized (Pirhonen *et al.* 1991). The *E. coli* control strain used was MC4100 (Casadaban 1976).

To obtain high-level constitutive expression of *lacZ* in *E. c.* subsp. *carotovora,* the plasmid pTK76 was introduced into the strains used by bacteriophage T4 transduction (Pirhonen and Palva 1988). This plasmid carries an *ompR-lacZ* translational fusion from *E. coli.* For plant inoculations, the bacteria were grown in L medium (Miller 1972) with the appropriate antibiotic, and the cells were harvested by centrifugation and resuspended into 10 mM $MgSO_4$. The acellular enzyme preparations were done as follows: for *E. c.* subsp. *carotovora* culture filtrates the wild-type strain SCC3193 and the $Exp^-$ mutant strain SCC3065 were grown to stationary phase at 28° C in L medium supplemented with 0.5% sodium polypectate (PGA; Sigma P-1879; Sigma Chemical Co., St. Louis, MO), the cells removed by centrifugation, and the supernatant sterilized by filtration. For preparations of individual exoenzymes, the *E. c.* subsp. *carotovora* genes cloned into pUC18 were introduced into *E. coli* strain HB101 (Boyer and Roulland-Dussoix 1969), the strains grown to midlogarithmic phase at 37° C in L Ap medium, and the enzymes released from the *E. coli* periplasm by osmotic shock as described (Palva 1978). These enzyme preparations were filter-sterilized and appropriate dilutions were sprayed onto plants. The enzyme activities were determined and the units defined as previously (Pirhonen *et al.* 1991). The genes encoding endopolygalacturonase, *pehA* (Saarilahti *et al.* 1990a), and cellulase, *celS* (Saarilahti *et al.* 1990b), have been described. The strain SCC3193 produces four major secreted isoforms of endopectate lyase (Pel) designated PelA, B, C, and D (our unpublished results). The corresponding genes have been cloned (our unpublished results) and the clones were used for production of the enzymes in *E. coli.*

**DNA and RNA preparations and Northern blot analysis.**

Plasmid DNA was isolated according to Birnboim and Doly (1979). Other standard DNA techniques were according to Maniatis *et al.* (1982). Total RNA from plant leaf tissue was isolated as described by Verwoerd *et al.* (1989). For Northern blot analysis 10 $\mu$g of RNA was loaded onto a denaturing formaldehyde-agarose gel (1%) and blotted after electrophoresis onto nitrocellulose filters (Ausubel *et al.* 1987). Nick-translated probes were prepared as described by Maniatis *et al.* (1982) from an isolated β-glucanase cDNA fragment corresponding to the *gnl* β-1,3-glucanase (De Loose *et al.* 1988; Castresana *et al.* 1990). The cDNA clone was kindly provided by Dirk Inzé (Laboratorium voor Genetica, Rijksuniversiteit Gent, Belgium). The hybridization and washing conditions were according to Maniatis *et al.* (1982).

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