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Erwinia carotovora subsp. *carotovora* Pectic Enzymes: *In Planta* Gene Activation and Roles in Soft-Rot Pathogenesis

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Pectic enzyme expression in *Erwinia carotovora* subsp. *carotovora* was studied on potato tuber slices using a membrane-separated system. Accumulation of mRNAs was sequential for *exo*-pectate lyase (*exo*-PL), *endo*-PL, and *endo*-polygalacturonase, respectively, and reached maxima by 6-12 hr. Induction kinetics *in vitro* with polygalacturonic acid differed from *in planta* kinetics. Accumulations of mRNA were correlated with rotting; under conditions incompatible with pathogenesis, induction was reduced, and *exo*-PL expression was delayed. Healed slices were resistant to rotting, but challenging *E. c.* subsp. *carotovora* were activated for transient *endo*-PL expression. This suggests that enzyme induction involves cell wall degradation products. Pretreatment of slices with *Escherichia coli* expressing *E. c.* subsp. *carotovora exo*-PL increased *in planta* accumulation of mRNAs for all three pectic enzymes in *E. c.* subsp. *carotovora* applied as a subsequent challenge, indicating that *exo*-PL reaction products activate other pectic enzyme genes. *Endo*-PL, expressed from *E. coli*, induced host accumulation of mRNAs for phenylalanine ammonia-lyase, a marker for plant defense-responses. These results support the hypothesis that pectic enzymes affect virulence by regulating pectic enzyme levels and affect host responses by inducing defense genes.

Erwinias produce diverse pectic enzymes including *endo*- and *exo*-pectate lyases (PLs), *endo*- and *exo*-polygalacturonases (PGs), pectin lyase, pectin methylesterase, and oligogalacturonate lyase (reviewed in Collmer and Keen 1986 and in Kotoujansky 1987). The number of enzymes, increased by numerous isozymes, probably reflects the complex pectic structures in plant cell walls and the wide host range of the pathogen and suggests that the interactions of pectic enzymes with plant tissue are complicated. Different catalytic properties or substrate preferences and/or differential expression of these enzymes may contribute to this complexity (Collmer and Keen 1986; Kotoujansky 1987).

Expression of pectic enzymes is important in soft-rot pathogenesis caused by erwinias (Collmer *et al.* 1982; Collmer and Keen 1986). Zucker and his colleagues showed that nonpathogenic bacteria synthesized pectic enzymes constitutively, whereas their synthesis in pathogenic bacteria was inducible (Zucker *et al.* 1972; Zucker and Hankin 1970). Since then, *in vitro* but not *in planta* regulation of pectic enzymes has been studied in soft-rot erwinias (Collmer *et al.* 1982; Reverchon and Robert-Baudouy 1987). In general, pectic enzyme synthesis is induced by isolated plant cell walls, enzyme substrates (pectate polymers and oligomers), and intermediates of pectate degradation pathways (Collmer and Bateman 1981,1982; Collmer *et al.* 1982) including saturated digalacturonate, unsaturated digalacturonate, 2-keto-3-deoxygluconate, 2,5-diketo-3-deoxygluconate, and 5-keto-4-deoxygluconate. *In vitro* studies show that pectic enzyme regulation is complex and characterized by multiple levels of genetic control (reviewed in Kotoujansky 1987), various sensitivities of one enzyme to different inducers (Condemine *et al.* 1986; Reverchon and Robert-Baudouy 1987), and various efficiencies for one inducer with different enzymes (Collmer *et al.* 1982).

In planta expression of pectic enzymes in pathogenesis has received very little attention. This is primarily due to difficulties in recovering sufficient quantities of bacteria from rotting tissue. For this reason, we developed a membrane-separated system to facilitate isolation of bacterial cells apart from soft-rotted potato (*Solanum tuberosum* L.) tubers for subsequent analysis of bacterial gene transcription (Yang *et al.* 1989). We showed that similar tissue maceration and induction of bacterial and plant gene expression occurs in both the membrane-separated and direct-contact interactions. Potato tubers are universally susceptible to soft rot albeit at different levels (Kelman *et al.* 1987); even the *S. tuberosum-S. brevidens* Phil. bacterial soft-rot resistant hybrids of Austin *et al.* (1988) develop rotting under some conditions. Therefore, in this paper, a compatible interaction (*sensu* Talboys *et al.* 1973) results in massive rotting that is not limited by host responses. An incompatible interaction occurs when either no rotting results or rotting is limited, presumably by general resistant responses of the host moderated by environmental conditions. Moisture is a major factor determining compatible (saturated moisture) versus incompatible (limited moisture) interactions (Yang *et al.* 1989).

Six or more pectolytic enzymes are produced *in vitro* (Roberts *et al.* 1986b) and at least two *endo*-pectate lyases (pIs 9.5 and 10.5) are produced *in planta* (Yang *et al.* 1989) by *Erwinia carotovora* subsp. *carotovora* (Jones) Bergey *et al.* strain ECl4. In this study, we utilized the membrane-separated system to examine *in planta* expression of *E. c.* subsp. *carotovora* genes encoding *endo*-PL (EC 4.2.2.2), *exo*-PL (EC 4.2.2.9), and *endo*-PG (EC 3.2.1.15), previously

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shown to be associated with soft-rot pathogenicity (Roberts *et al.* 1986b). Specifically, we address: 1) Which genes are expressed *in planta*? 2) Is their induction associated with disease development? 3) Are these genes under coordinate regulation? 4) Is their *in planta* induction similar to *in vitro* induction? 5) Are they expressed under incompatible conditions? 6) What molecules are inducers *in planta*?

Reaction products of pectic enzymes activate plant defense responses (reviewed in Hahn *et al.* 1989 and Ryan 1987). Davis *et al.* (1984, 1986) show that *E. c.* subsp. *carotovora endo*-PL elicits accumulation of soybean (*Glycine max* (L.) Merr.) phytoalexins by releasing oligogalacturonides from host plant cell walls. In French bean (*Phaseolus vulgaris* L.), accumulation of phytoalexins elicited by challenge with *Colletotrichum lindemuthianum* (Sacc. & Magn.) Briosi & Cav. or fungal cell wall crude extracts is attributed to the activation of plant genes encoding biosynthetic enzymes (Cramer *et al.* 1985; Dixon *et al.* 1986). We have shown that inoculation with *E. c.* subsp. *carotovora* induces two plant defense-related genes, phenylalanine ammonia-lyase (PAL) and DL-3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) in potato tubers, which are involved in responses to soft rot (Yang *et al.* 1989,1991). Pectic enzymes are important factors in soft-rot pathogenesis. We ask here if pectic enzymes are also involved in plant defense gene activation. Because *Escherichia coli* (Migula) Castellani & Chalmers does not induce potato tuber defense responses (Yang *et al.* 1989), *E. coli* strains containing *E. c.* subsp. *carotovora* genes cloned on plasmids were used to determine which pectic enzymes create reaction products that may induce defense responses.

MATERIALS AND METHODS

Plasmids, bacterial strains, and plant materials. Construction of plasmids pDR1 (encoding *endo*-PL) and pDR30 (encoding *exo*-PL and *endo*-PG) was described by Roberts *et al.* (1986a,b); plasmid pZY12 was constructed for this research by cloning a 1.9-kb *Bam*HI-*Hin*cII fragment encoding only *exo*-PL from pDR30 into pUC118 (Fig. 1). *E. c.* subsp. *carotovora* strain EC14, *E. coli* strains DH5α, HB101, or HB101 or DH5α containing pDR1, pDR30, or pZY12 were maintained on plate count agar (Difco Laboratories, Detroit, MI) supplemented with ampicillin (30 μg/ml; Sigma, St. Louis, MO) when necessary. For plant inoculation, bacteria were cultured in tryptic soy broth (Difco) supplemented with appropriate antibiotics and incubated with orbital shaking (250 rpm) at 30° C. Following overnight growth, the bacterial suspension was diluted (1:100) in minimal medium supplemented with 1% (v/v) glycerol (Yang *et al.* 1989) and incubation with shaking was continued for 12 hr or until a turbidity of 1.0 at 550 nm was achieved. Potato tubers (*S. tuberosum* 'Russet Burbank') were purchased from a local market. Before inoculation, tubers were surface-disinfested and incubated at 30° C overnight as described previously (Yang *et al.* 1989).

Inoculation and harvest of bacterial cells and plant tissues. For analysis of *in planta* expression of bacterial genes, *E. c.* subsp. *carotovora* grown in minimal medium plus glycerol was inoculated (1.5×10^7 colony-forming units [cfu] in 600 μl) onto polysulfone membranes (0.2-μm pore size, GA-8S, Gelman Sciences, Ann Arbor, MI) placed on cut surfaces of potato tuber slices under conditions compatible or incompatible with soft-rot development as described for the membrane-separated system (Yang *et al.* 1989). To determine if pectic enzyme genes can be induced on wound-healed surfaces, tubers were sliced and incubated at 30° C for 3 days before inoculation. To test if *E. c.* subsp. *carotovora exo*-PL reaction products induce pectic enzymes, slices were preinoculated directly (without membranes) with *E. coli* or *E. coli* expressing *exo*-PL from plasmid pZY12. Preinoculated slices were incubated at 30° C under compatible conditions for 6 hr before inoculation with *E. c.* subsp. *carotovora* by using membranes. At various times after inoculation, bacteria were harvested from the membranes, frozen in liquid nitrogen, and stored at −75° C for subsequent RNA isolation.

To analyze induction of plant defense-related genes by pectic enzymes, tuber slices were inoculated directly with a 1.0-ml suspension of *E. coli* strain HB101 or *E. coli* containing pDR1 or pDR30 (10^8 cfu). As a control for defense induction, 300 μl of *E. c.* subsp. *carotovora* in suspension (3×10^7 cfu) was used to inoculate other slices. Slices were inoculated with 1.0 ml of minimal medium plus glycerol as a wound control. Six or 9 hr after inoculation, the top 1.0 mm of tuber tissue was sliced off, frozen immediately in liquid nitrogen, and stored at −75° C for subsequent RNA isolation.

Induction of pectic enzymes by polygalacturonic acid. *E. c.* subsp. *carotovora* cells grown in minimal medium

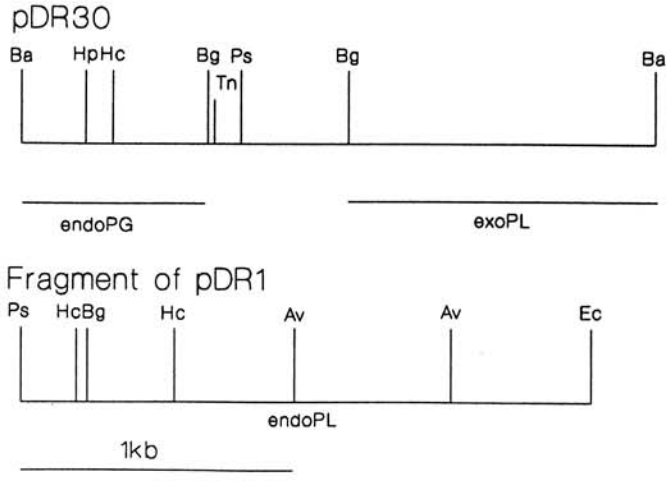

Fig. 1. Gene probes for *endo*-polygalacturonase (*endo*PG), and *exo*- and *endo*-pectate lyases (*exo*PL, *endo*PL) were derived from *Erwinia carotovora* subsp. *carotovora* DNA fragments, diagramed in this figure, cloned in plasmids pDR30 and pDR1 (Roberts *et al.* 1986a,b). Endonuclease restriction sites (Av = *Ava*I, Ba = *Bam*HI, Bg = *Bgl*II, Ec = *Eco*RI, Hc = *Hin*cII, Hp = *Hpa*I, Ps = *Pst*I) are indicated. Activity of *exo*-PL and *endo*-PG is abolished by a Tn*5* (Tn) insertion. Because the genes may both extend into the *Bgl*II-*Bgl*II fragment, we used the 1.1-kb *Bam*HI-*Bgl*II fragment for the *exo*-PL gene probe and the 0.7-kb *Bam*HI-*Bgl*II fragment for the *endo*-PG gene probe. The 2.1-kb *Pst*I-*Eco*RI fragment of pDR1 was used as probe for *endo*-PL; this fragment expresses the enzyme. The bar at the bottom indicates 1 kb.

plus glycerol (OD_{550nm} = 1.0) were pelleted at 3,000 × *g* for 10 min at 4° C and resuspended in the same volume of minimal medium plus 0.8% polygalacturonic acid sodium salt (w/v; Sunkist). At various times of incubation at 30° C with orbital shaking (250 rpm), bacterial cells from 1.5 ml of culture were pelleted by centrifugation at 10,000 × *g* for 1.0 min at room temperature, frozen immediately in liquid nitrogen, and stored at −75° C for later RNA isolation.

RNA isolation and RNA:DNA hybridization. Bacterial and plant total RNAs were isolated as described previously (Yang *et al.* 1989). For RNA:DNA hybridization analyses, 10 μg of bacterial RNA was treated with RNase-free DNase I (Boehringer Mannheim Biochemicals, Indianapolis, IN), separated by electrophoresis on 1% agarose gels, transferred to a Nytran membrane (Schleicher and Schuell, Inc., Keene, NH), and hybridized to ^{32}P-labeled DNA probes as described previously (Yang *et al.* 1989). To re-use the membrane, the hybridized probe DNA was stripped off by incubating the membrane in a 70% formamide and 6× SSPE (1.08 M sodium chloride, 60 mM monobasic sodium phosphate, and 6 mM EDTA at pH 7.4 [Sambrook *et al.* 1989]) at 65° C for 40 min. Each time the probe was stripped off, the membrane was autoradiographed for two days to assure that the probe was completely removed. As an internal control for constitutive expression of bacterial mRNA, the membranes were also probed with a 5.0-kb *Eco*RI fragment of pN01001 encoding *E. coli* ribosomal proteins (S5, L18, L6, S8, S14, S17, L29, L16, and S3) (Cerretti *et al.* 1983). Probe preparation and labeling were as described (Yang *et al.* 1989). Following autoradiography, a semi-quantitative method for analyses of the films was selected; they were scanned with a densitometer, and mRNA levels were presented as percentage of maximum density for each autoradiogram. Sizes for bacterial mRNAs hybridizing to the gene probes were estimated using a 1-kb RNA molecular mass standards (0.16–1.77 kb and 0.24–9.5 kb RNA ladders, Bethesda Research Laboratories [BRL], Gaithersburg, MD). Using this method, the *endo*-PL probe was found in a previous study to hybridize to a 1.4-kb class of RNA (Yang *et al.* 1989).

PAL mRNA levels were determined by slot blot hybridization using a 1.8-kb *Pst*I fragment of PAL cDNA from bean (Edwards *et al.* 1985). Five micrograms of total potato RNA was denatured by glyoxalation (Williams and Mason 1985) and blotted onto Nytran membrane in a Millipore slot apparatus (Millipore Corp., Bedford, MA). PAL DNA probe and hybridization conditions were described previously (Yang *et al.* 1989).

RESULTS

Characterization of gene probes. The origins of the gene probes are shown in Figure 1. Genes for *endo*-PL and *exo*-PL are related by nucleotide sequence analyses (L. S. Antonov and V. K. Stromberg, unpublished data), respectively, to genes mediating production of the Pelbc family of extracellular *Erwinia* enzymes and to the family of intracellular pectate lyases (described in Hinton *et al.* 1989). In this study, expression of *exo*-PL by the 1.9-kb *Bam*HI-*Hin*cII fragment of pDR30 cloned in pZY12 establishes that the gene encoding that enzyme resides in that fragment as suggested by Hinton *et al.* (1989). Because *exo*-PL and *endo*-PG activity is abolished by a Tn*5* insertion or deletion of the central 0.5-kb *Bgl*II fragment, the genes encoding these enzymes may overlap, their promoters may overlap, or they may be expressed from an operon. Preliminary sequence analyses (V. K. Stromberg, unpublished data) suggest that the gene encoding *endo*-PG bears only slight nucleotide similarity to a PG from *E. c.* subsp. *carotovora* (Hinton *et al.* 1990). This may not be surprising because *E. c.* subsp. *carotovora* may also have multiple PGs as does the closely related soft-rot bacterium, *E. c.* subsp. *atroseptica* (George *et al.* 1991). The *exo*-PL and *endo*-PG probes hybridized to 1.8- and 1.3-kb mRNA, respectively, demonstrating that these genes are not encoded by a polycistronic message characteristic of a single operon. This observation was repeated once for each probe, and representative data are shown in Figure 2A and B. Both mRNAs accumulated to higher levels in cells recovered from tuber slices compared to cells grown in minimal medium plus glycerol (Fig. 2A,B). In a previous study (Yang *et al.* 1989), the *endo*-PL probe was found to hybridize to a 1.4-kb mRNA species.

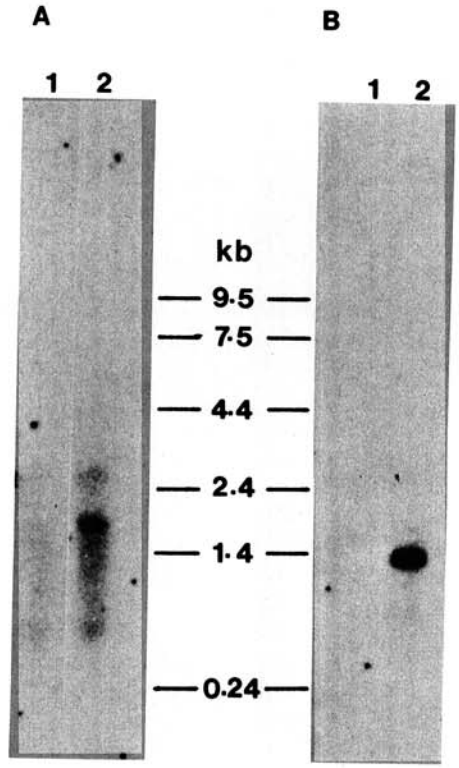

Fig. 2. RNA:DNA hybridization analyses of *exo*-pectate lyase (PL), and *endo*-polygalacturonase (PG) mRNAs. Total RNA was isolated from *Erwinia carotovora* subsp. *carotovora* grown in minimal medium plus glycerol (1A,B) or inoculated on potato tuber slices in the membrane-separated system (see text) (2A,B). Ten micrograms of total RNA was separated by electrophoresis on a 1.2% agarose gel, transferred to and immobilized on a Nytran membrane, and hybridized to ^{32}P-labeled *exo*-PL (**A**) and *endo*-PG (**B**) DNA probes. An RNA ladder (Bethesda Research Laboratories, Gaithersburg, MD) was used to determine molecular size (kb).

Kinetics of *in planta* induction of three pectic enzyme mRNAs. Previously, we showed that mRNA levels and enzyme activity for *E. c.* subsp. *carotovora endo*-PL were induced *in planta* in either membrane-separated or direct-contact interactions (Yang *et al.* 1989). Kinetics of *endo*-PL, *exo*-PL, and *endo*-PG mRNAs accumulations were studied during disease development utilizing the membrane-separated system. This experiment was repeated twice with similar results; representative data are presented in Figure 3A. Under compatible conditions, levels of *endo*-PL, *exo*-PL, and *endo*-PG mRNAs were induced within 3 hr after inoculation, and accumulations reached maxima between 6 and 12 hr and essentially returned to basal levels by 24 hr. For individual mRNAs, maximal accumulation occurred at different times after inoculation, suggesting that the three pectic enzymes appear sequentially *in planta*. *Exo*-PL had the highest basal level of mRNA production at time zero and accumulated mRNA most rapidly, peaking at 6 hr. This was followed by *endo*-PL, which reached a peak at 9 hr. Accumulation of *endo*-PG was not significant until 6 hr and did not reach its maximum until 12 hr after inoculation, continuing with near maximal levels through 15 hr. Linear increase in tissue maceration occurred between 6 and 12 hr.

***In vitro* induction of pectic enzymes by polygalacturonic acid.** To determine if *in planta* expression of pectic enzymes is similar to or distinct from *in vitro* expression, kinetics for *in vitro* induction of *endo*-PL, *exo*-PL, and *endo*-PG mRNAs by polygalacturonic acid were examined. This experiment was repeated twice with similar results; representative data are shown in Figure 3B. *In vitro* induction of all three mRNAs was more rapid and more transient compared to *in planta* induction. Both *endo*-PG and *exo*-PL mRNAs were induced to maxima during the early exponential increase of bacterial growth (within 60 min after adding inducer); their mRNA levels fell to basal levels by 4 hr. Levels of *endo*-PL mRNA increased by 60 min and reached peak levels within 4 hr during the late exponential growth of the bacterium. *In vitro* expression of *endo*-PG was induced simultaneously with *exo*-PL and before *endo*-PL, while *in planta*, *endo*-PG induction was the latest to reach maximum accumulation of mRNAs among the three enzymes.

Expression of pectic enzyme mRNAs under incompatible conditions. Reduced moisture limits maceration (Yang *et al.* 1989). In Figure 4, we compared levels of expression of *endo*-PL, *exo*-PL, and *endo*-PG mRNAs under both high moisture (compatible) and low moisture (incompatible) conditions. All three mRNAs were induced under incompatible conditions as well as compatible conditions, but the mRNA accumulations were reduced markedly under incompatible conditions except for *endo*-PL. Under incompatible conditions, expression of *exo*-PL was delayed past 3 hr after inoculation, whereas accumulations for *endo*-PL and *endo*-PG mRNAs occurred at times similar to those observed under compatible conditions. Levels of *endo*-PL mRNA accumulation at 24 hr were higher in the incompatible compared to the compatible interactions; in the compatible interaction, *endo*-PL mRNA levels returned to basal levels by 24 hr. This experiment was repeated once; representative data are shown in Figure 4.

Wound-healed potato tuber tissues are resistant to *E. c.* subsp. *carotovora* (Tripathi and Gupta 1983). Because of its central role in soft rot (Collmer *et al.* 1982; Collmer and Keen 1986; Kotoujansky 1987), we wanted to learn if *endo*-PL might be induced by healed tissues as well as by wounded tissues. Analyses of the induction of pectic enzyme on tuber slices wound-healed for three days and completely resistant to maceration by *E. c.* subsp. *carotovora* showed that *endo*-PL mRNA was transiently induced to maximal levels at 4 hr after inoculation (data not shown).

Induction of pectic enzyme genes by their own reaction products. To determine if *E. c.* subsp. *carotovora* pectic enzyme reaction products are involved in the induction of pectic enzymes, tuber slices were preinoculated directly with *E. coli* expressing the *E. c.* subsp. *carotovora exo*-PL gene from plasmid pZY12. For controls, *E. coli* lacking the plasmids or containing only plasmid pUC118, the vector for cloned *E. c.* subsp. *carotovora* DNA, were used. Following incubation at 30° C for 6 hr, the preinoculated tuber slices were inoculated with *E. c.* subsp. *carotovora* using the membrane-separated system. Preinoculation with *E. coli* expressing *E. c.* subsp. *carotovora exo*-PL resulted in significant increases in mRNA levels for *exo*-PL, *endo*-PL, and *endo*-PG over *E. coli* alone. This experiment was repeated once; representative data are shown in Figure 5. These results indicate that *exo*-PL produces reaction products that activate expression of its own and other pectic enzyme genes during pathogenesis. Increases in *endo*-PG mRNA accumulation levels induced by *exo*-PL were most prominent among the three mRNAs examined, suggesting that *exo*-PL reaction products including unsaturated digalacturonate are efficient inducers of *endo*-PG. No maceration was caused by *E. coli* alone or the expression of the *E. c.* subsp. *carotovora* genes in *E. coli*; this result is similar to the observations of Roberts *et al.* (1986b).

Activation of plant defense-related genes by pectic enzymes. To determine if *E. c.* subsp. *carotovora* pectic enzymes are involved in the induction of plant defense responses, potato tuber slices were inoculated directly with *E. coli* expressing *E. c.* subsp. *carotovora* pectic enzyme genes from plasmids. This experiment was repeated twice; representative data are shown in Figure 6. Inoculation with *E. coli* alone did not increase PAL mRNA levels more than the wounding, which resulted from slicing the tuber. Plasmid pDR1 in *E. coli*, encoding *E. c.* subsp. *carotovora endo*-PL, caused an increase in potato PAL mRNA which at 9 hr was similar in intensity to wild-type *E. c.* subsp. *carotovora* after 6 hr. At 6 hr (data not presented), the *E. coli*/pDR1 response was not as strong as the *E. c.* subsp. *carotovora* response at the same time. Plasmid pDR30 in *E. coli*, encoding *endo*-PG and *exo*-PL, did not induce accumulation of PAL mRNA.

DISCUSSION

***In planta* induction of pectic enzymes is sequential.** Our studies, using a membrane-separated *E. c.* subsp. *carotovora*-potato interaction system, suggest that *in planta*

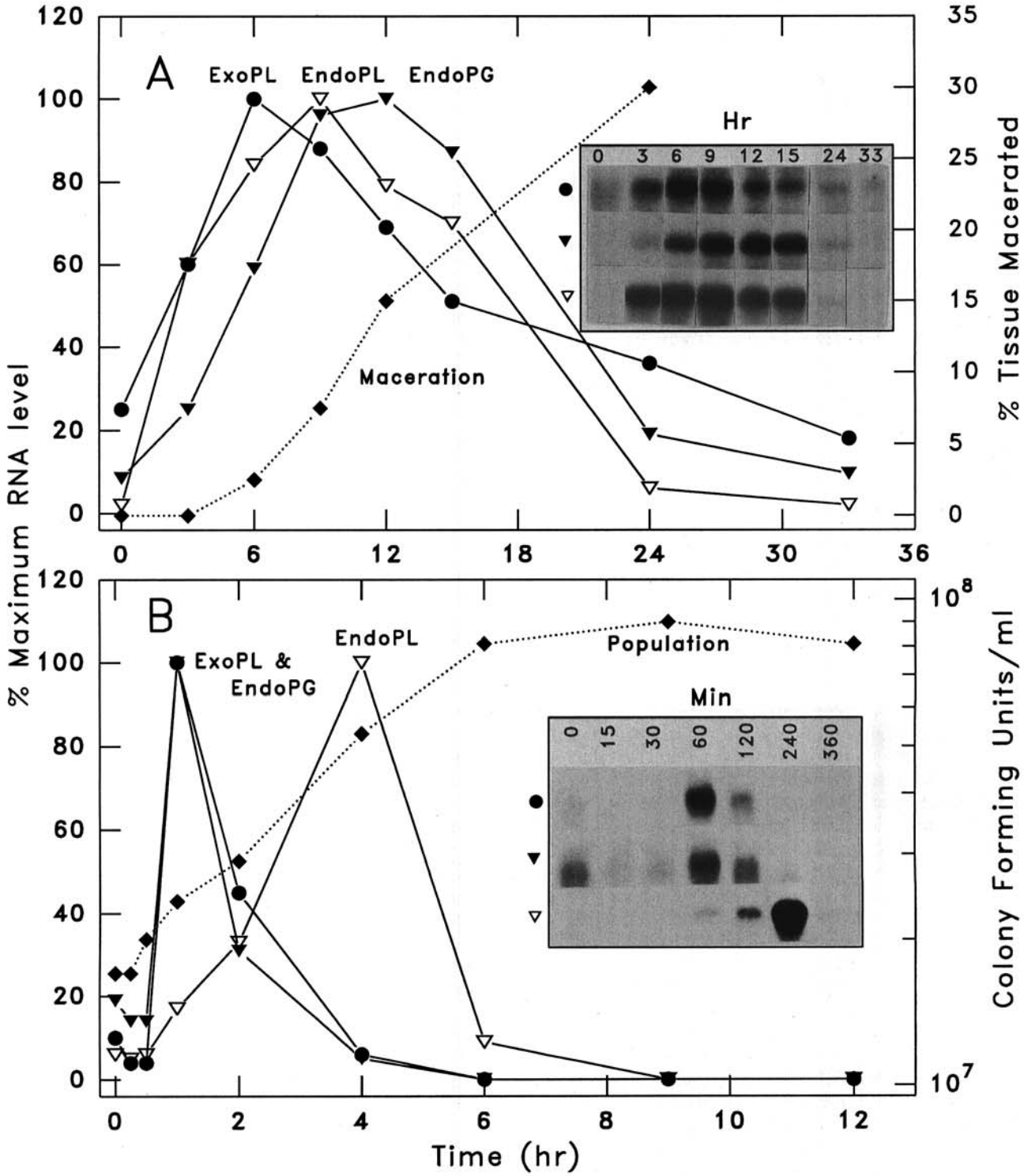

Fig. 3. RNA:DNA hybridization analyses of the *in planta* and *in vitro* kinetics of transcriptional induction for *exo*-pectate lyase (PL; filled circles), *endo*-PL (open triangles) and *endo*-polygalacturonase (PG; filled triangles) mRNAs. **A,** *in planta*: bacterial RNAs were isolated from *Erwinia carotovora* subsp. *carotovora* cells induced under compatible conditions in the membrane-separated system (see text). Percentage of tuber tissue macerated (filled diamonds) was determined by weight loss after washing rotted tissue off tuber slices standardized for weight loss due to metabolic activities at various times following inoculation. **B,** *in vitro*: *E. c.* subsp. *carotovora* cells were grown in minimal medium (MM) plus glycerol to OD_{550} 1.0 and resuspended in MM plus polygalacturonic acid. At various times after resuspension, cells were harvested, and total RNA was isolated. *E. c.* subsp. *carotovora* populations (cfu; filled diamonds) were also determined at the harvest times. Following hybridization to a probe and autoradiography, that probe was stripped off both RNA blots (A and B), and the blots were reprobed separately with the other two probes. Autoradiograms (shown in the insets) were scanned by densitometer to provide semiquantitative estimates of the intensity of the DNA:RNA hybridizations. Relative mRNA accumulation levels were presented as percentage of maximum density.

accumulation of mRNAs for three pectic enzymes is sequential (*exo*-PL, *endo*-PL, and *endo*-PG) rather than simultaneous and that they are encoded on separate small transcripts rather than on polycistronic message. This suggests that genes for pectic enzymes may be regulated separately rather than by a global mechanism. Accumulation profiles for these transcripts induced in *E. c.* subsp. *carotovora* during pathogenesis are distinct from accumulation profiles *in vitro*, indicating that *in planta* expression of pectic enzymes is different from *in vitro* expression. Sequential production of cell wall-degrading enzymes (pectic enzymes preceding cellulase) occurs in fungal pathogens (Cooper 1983). Several hypotheses for the biological significance for sequential induction are put forth. First, pathogenesis by *E. c.* subsp. *carotovora* may require that enzymes be expressed sequentially. Second, separate and sequential induction may allow a wide host-range pathogen such as *E. c.* subsp. *carotovora* to mobilize pectic enzyme expression to accommodate pathogenesis on a wide variety of hosts. Third, due to varying efficiencies of different inducers for pectic enzymes (Collmer *et al.* 1982; Reverchon and Robert-Baudouy 1987), sequential induction may imply cascade regulation (i.e., reaction products of first enzyme preferentially induce the next enzyme, and so on).

Role of pectic enzyme expression in soft-rot pathogenesis. Regulation of pectic enzymes probably plays an important role in soft-rot pathogenesis (Collmer *et al.* 1982; Collmer and Keen 1986). Our studies provide *in planta* evidence supporting this hypothesis. First, increases in gene transcription levels for the pectic enzymes examined were associated with increases in tissue maceration. Second, tissue maceration slows down as the expression of these genes decreases, presumably due to self-catabolite repression (Collmer and Bateman 1981). Third, induction of all three enzymes is reduced under conditions incompatible with soft rot compared to conditions compatible with rot. Other regulatory mechanisms are probably important in pathogenesis and remain to be examined. For instance, other research suggests that low oxygen levels effect changes in plant defense responses (Antonov *et al.* 1990; Rumeau *et al.* 1990; Vayda and Schaeffer 1988) but do not effect production of pectic enzymes by the pathogen (Maher and Kelman 1983).

Pectic enzymes are involved in plant-bacterial communication. Our data also provide background for speculation concerning the hypothesis that pectic enzyme reaction products may function in host defense responses as well as in pathogenesis (Cervone *et al.* 1989; Hahn *et al.* 1989). Significant levels of *endo*-PL mRNA were maintained through 24 hr under conditions incompatible with soft rot but not under conditions compatible with rot. This result suggests that *endo*-PL may be available to produce oligogalacturonides active as inducers of host defense responses and is consistent with our earlier finding that PAL mRNA

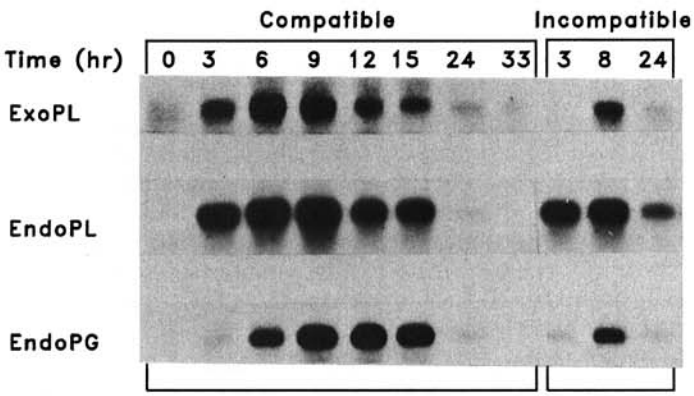

Fig. 4. Comparison of transcription for *exo*-pectate lyase (PL), *endo*-PL, and *endo*-polygalacturonase (PG) in compatible and incompatible interactions. Total bacterial RNAs were isolated from *Erwinia carotovora* subsp. *carotovora* cells inoculated on potato tubers in the membrane-separated system (see text) under compatible conditions favoring rotting or under incompatible conditions favoring resistance to rot where moisture was limited (see text). Times 0 represents the mimimal broth plus glycerol-grown cells used for inoculation. RNA:DNA hybridization analyses were performed as described in Figure 3.

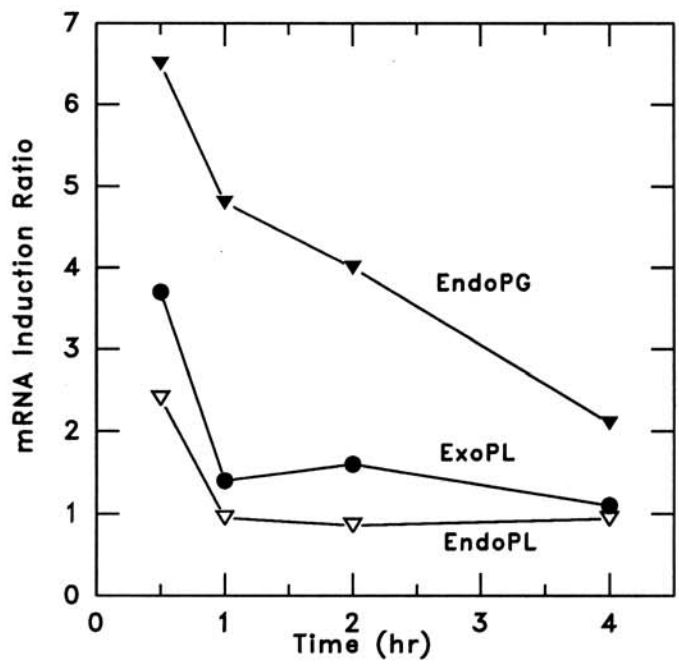

Fig. 5. *In planta* accumulation of mRNAs for *Erwinia carotovora* subsp. *carotovora* pectic enzymes induced by tuber exposure to *E. c.* subsp. *carotovora*, *exo*-pectate lyase (PL). Tuber slices were inoculated directly with *Escherichia coli* strain DH5α or *E. coli* DH5α expressing *E. c.* subsp. *carotovora exo*-PL from plasmid pZY12. Following incubation at 30° C for 6 hr, the slices were inoculated with *E. c.* subsp. *carotovora* as in the membrane-separated system (see text). *E. c.* subsp. *carotovora* cells were harvested from the membranes at the times indicated, and DNA-free total RNA was isolated. Autoradiograms were scanned by a densitometer. Five micrograms of total *E. c.* subsp. *carotovora* RNA was immobilized onto a Nytran membrane using a Millipore slot blot apparatus and hybridized to *endo*-PL DNA probe (open triangles). The blot was stripped and reprobed separately with *exo*-PL (filled circles) and *endo*-polygalacturonase (filled triangles) DNA probes as described in Figure 3. As a control for constitutive RNA expression and loading, the blot was stripped and probed with a 5.0-kb *Eco*RI DNA fragment encoding *E. coli* ribosomal proteins (Cerretti *et al.* 1983). Levels of specific mRNAs were standardized for internal variation versus the ribosomal protein standards. Degrees of induction resulting from *exo*-PL pretreatment were presented as induction ratio, a ratio of specific mRNA levels for *E. c.* subsp. *carotovora* inoculated on tuber slices with *exo*-PL pretreatment versus those for *E. c.* subsp. *carotovora* inoculated on tuber slices without *exo*-PL pretreatment.

continues to increase through 12 hr after *E. c.* subsp. *carotovora* inoculation under incompatible conditions but not under compatible conditions (Yang *et al.* 1989). That PAL mRNA levels were higher for *E. c.* subsp. *carotovora*-inoculated tubers at 6 hr than at 9 hr may indicate that *endo*-PL is not solely responsible for the induction of the host response.

E. c. subsp. *carotovora* pectate lyase(s) releases oligosaccharide elicitors from plant cell walls that induce plant defense responses, including phytoalexin accumulation in soybean (Davis *et al.* 1984,1986). In those studies, the possibility that the actual inducer is a minor contaminant in oligogalacturonide or enzyme preparations cannot be eliminated (Collmer and Keen 1986). In our study, *E. coli* does not induce a plant defense response, but *E. coli* expressing a cloned *E. c.* subsp. *carotovora endo*-PL does induce PAL mRNA. Therefore, our results confirm the capacity of pectate lyase to elicit, probably via oligosaccharides, a plant defense response involving activation of defense-related genes. This is consistent with Davis and Ausubel's (1989) studies showing that crude preparations of *E. c.* subsp. *carotovora endo*-PL increased expression

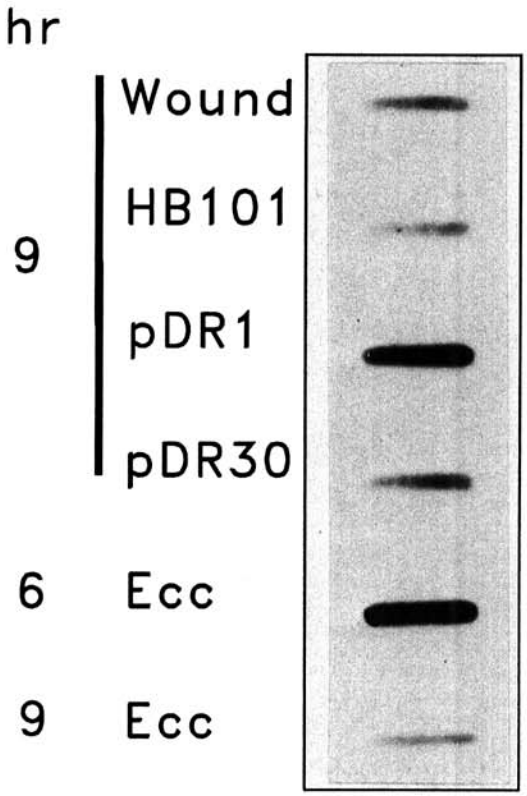

Fig. 6. Activation of potato phenylalanine ammonia-lyase (PAL) gene by *Erwinia carotovora* subsp. *carotovora* pectic enzymes. Potato tubers were sliced (row 1, top) and inoculated with *Escherichia coli* HB101 (row 2), or HB101 containing plasmid pDR1 encoding *E. c.* subsp. *carotovora endo*-PL (row 3), or pDR30 encoding *E. c.* subsp. *carotovora exo*-PL and *endo*-PG (row 4) and incubated at 30° C for 9 hr. Tuber slices inoculated with *E. c.* subsp. *carotovora* (Ecc) were incubated for 6 hr (row 5) or 9 hr (row 6). Following incubation, the top 1 mm of tuber tissue was sliced off and used for RNA isolation. PAL mRNA levels were determined by RNA slot blot hybridization according to Yang *et al.* (1989).

of a number of plant defense genes including PAL and 4-coumarate:coenzyme A ligase genes. Our results also suggest that *endo*-PL reaction products may be responsible at least in part for the increased PAL mRNA and enzyme activity levels induced by *E. c.* subsp. *carotovora* (Yang *et al.* 1989). Although complete digestion of pectate by *endo*-PL results in unsaturated trimers to hexamers (Roberts *et al.* 1986b), incomplete digestion would result in production of oligomers with eight to 12 galacturonic acid residues, large enough to act as plant defense elicitors (Davis *et al.* 1986). Expression of *endo*-PG and *exo*-PL from plasmid pDR30 did not induce PAL mRNA, although *endo*-PG from fungal pathogens triggers plant defense responses (Cervone *et al.* 1989; Collmer and Keen 1986). *E. c.* subsp. *carotovora endo*-PG expressed by *E. coli* may lack the ability to release active elicitor or may not release sufficient amounts of elicitors to induce defense response. Because small oligogalacturonides do not have elicitor activity (Davis *et al.* 1986; Hahn *et al.* 1989), *exo*-PL, which produces unsaturated dimers and trimers (Roberts *et al.* 1986b), is not expected to induce defense responses, but it may cleave elicitor-active large oligogalacturonides to nonelicitor-active small oligogalacturonides. This is consistent with our results showing that *exo*-PL mRNA accumulation was rapid under compatible conditions but was absent early in the interaction under incompatible conditions.

On the other hand, *exo*-PL may also generate pectic oligomers as inducers of pectic enzymes. Reaction intermediates are effective for inducing pectic enzymes *in vitro*; the same mechanism has been proposed for *in planta* induction (Collmer and Bateman 1981,1982; Collmer *et al.* 1982; Collmer and Keen 1986; Stack *et al.* 1980). We found that on wound-healed tuber slices, *endo*-PL mRNA was induced transiently. Because wound-healed tuber slices were resistant to degradation by *E. c.* subsp. *carotovora* enzymes, this suggests that enzyme reaction products are required for continuing *in planta* induction. Transient accumulation of *endo*-PL mRNA is probably the result of constitutive inducers present in the wound-healed tuber slice or of trace amounts of plant cell wall fragments accessible to the pathogen. Our results indicate that *exo*-PL activates genes for production of *endo*-PG, *endo*-PL, and *exo*-PL presumably by releasing inducers. This may also explain why *Erwinia chrysanthemi* Burkholder *et al.* mutants deficient in oligogalacturonide lyase macerate plant tissue as well as the wild-type (Collmer and Keen 1986); *E. chrysanthemi exo*-PL expression could also produce inducers, thus, bypassing the need for oligogalacturonide lyase, which is thought to be required for generation of pectic enzyme inducers from pectic oligomers (Collmer and Bateman 1981).

Our results confirm that bacterial pectic enzymes are critical in plant-bacterial communication in erwinia soft rot. On one hand, enzymes such as *exo*-PL are involved in producing reaction intermediates as inducers to mediate their own induction. On the other hand, enzymes such as *endo*-PL activate plant defense responses by releasing oligogalacturonide elicitors from plant cell walls. This supports the hypothesis of Hahn *et al.* (1989) that bacterial

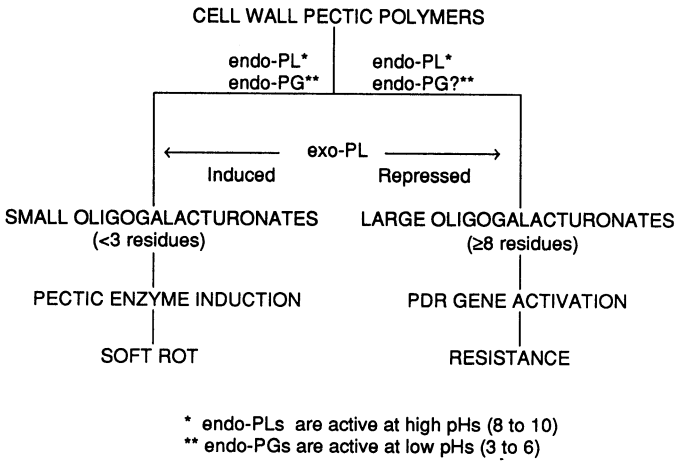

Fig. 7. A model for the role of pectic enzyme regulation in plant-bacterial communication. This model suggests that the dual functions of pectic enzymes in pathogenesis, cell-wall degradation and elicitor synthesis are mediated by the regulation of these enzymes. When the production of these enzymes is up-regulated, their function is shifted towards generation of elicitor-inactive oligogalacturonides (OGAs), which can be further catabolized to produce inducers of pectic enzymes. When pectic enzymes are down-regulated, low levels of *endo*-pectate lyase (PL) allow generation of elicitor-active OGAs that induce plant defense responses (PDR). Regulation of *exo*-PL and maybe *exo*-polygalacturonase (PG) is critical, particularly early in the interaction. Decreases in *exo*-PL shift the interaction towards defense activation; increases in *exo*-PL shift the interaction towards pathogenesis. Other factors, including pH (≤6) of plant surfaces and tissue or the pH (≥8) of damaged cells may also affect enzyme activity.

pectic enzymes have dual and apparently opposing functions: macerating plant tissues and triggering defense responses. Because elicitor activity depends on the number of galacturonic acid residues, the amount of elicitor may be determined by the efficiency of pectic decomposition (Hahn *et al.* 1989). Cervone *et al.* (1989) suggest that the incomplete digestion of pectate by *endo*-PL (optimal activity at pH 8–10) at the nonoptimal physiological pH of plant surfaces or tissues (pH 6 or lower) results in release of elicitor-active oligogalacturonides. However, their hypothesis did not consider the combined action of *endo*-PG (optimal activity pH ≤6) and *endo*-PL that, as indicated by our results, are both present at some periods during pathogenesis. Further, *E. coli* strains expressing high levels of a single *E. chrysanthemi endo*-PL successfully macerate plant tissues (Keen and Tamaki 1986), suggesting that high levels of *endo*-PL overcome plant defense responses. We propose that *in planta* expression of various pectic enzymes also plays an important role in shifting between the dual functions of pectic enzymes, thus affecting the outcome of soft-rot interactions (see Fig. 7). Studies using mutants that affect *in planta* expression of these enzymes would confirm this hypothesis. Furthermore, characterizations of *in planta* expression of other enzymes including various PL isozymes will provide important insight into the complex interaction of erwinia pectic enzymes with plants.

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