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Cloning of Genes Affecting Polygalacturonase Production in *Pseudomonas solanacearum*

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An extracellular polygalacturonase (PG) produced by the bacterial wilt pathogen *Pseudomonas solanacearum* induced rapid browning of potato tissue culture callus and was identical to a previously described browning factor. The structural gene for this enzyme was cloned from a genomic library of *P. solanacearum* K60 and mutagenized with Tn*3* carrying a β-glucuronidase reporter gene. The PG gene (called *pehA*) was located at the edge of the Vir2 cluster of *hrp* genes previously described by Christian Boucher and co-workers in strain GMI1000 of *P. solanacearum*. The mutagenized PG gene was marker-exchanged into the wild-type chromosome of strain K60; the resulting mutant strain no longer caused browning on callus but was still pathogenic on tobacco and eggplant and retained the ability to elicit a hypersensitive response on nonhost species. The mutant no longer produced the pI 9.0 enzyme encoded by *pehA*. In the wild-type strain, total extracellular PG activity increased about fivefold when bacteria were grown in the presence of tobacco leaf intracellular fluids and about 100-fold when bacteria were inoculated into tobacco leaves. Three Tn*5* mutants, one from strain B1 and two from strain K60, no longer browned callus or produced detectable PG activity in culture, but Southern blot analysis showed that in all three mutants the transposon was inserted into a common 4.5-kilobase region which is not in or near *pehA*. When these mutants were complemented with the corresponding wild-type fragment, the resulting *trans*-merodiploid strains produced about eight times the wild-type level of PG activity. This locus, called *pehR*, may encode a *trans*-acting positive regulator of PG production.

Additional keywords: bacterial wilt, pectinolysis, *Solanum phureja*.

Pseudomonas solanacearum E. F. Sm. is the causal agent of bacterial wilt disease of many economically important crop species worldwide. Although the exact physiological basis of wilting is not understood, several factors are suspected of playing a role in pathogenicity by this bacterium. Among these are bacterial production of extracellular polysaccharide (EPS), plant cell wall-degrading enzymes such as pectinases and cellulases, and plant growth substances such as indoleacetic acid and ethylene (Bonn *et al.* 1975; Husain and Kelman 1958; Phelps and Sequeira 1968; Roberts *et al.* 1988; Schell *et al.* 1988). These traits have been associated with pathogenicity in part because they are often lost or altered in spontaneous, nonpathogenic mutants of *P. solanacearum*. For example, the spontaneous mutant strain B1 isolated by Kelman (1954) is nonpathogenic, EPS$^-$, reduced in cellulase production, and an overproducer of polygalacturonase (PG). Several other phenotypes are affected as well.

Recently, molecular techniques have been used to examine the genetic basis of pathogenicity and virulence in *P. solanacearum*. Several regions of DNA have been identified that are required for both pathogenicity on host plants and induction of the hypersensitive response (HR) on nonhost plants (*hrp* genes) (Boucher *et al.*1987; Huang *et al.* 1990; Xu *et al.* 1988). Christian Boucher and co-workers have shown that a large (25-kilobase [kb]) cluster of *hrp* genes called the Vir2 region is located on a megaplasmid in many strains of *P. solanacearum* (Boucher *et al.* 1987) A second cluster of *hrp* genes was recently identified (Huang *et al.* 1990). The functions encoded by these genes remain unknown.

Marker exchange mutagenesis has been used to inactivate structural genes for PG and endoglucanase (a type of cellulase) in separate mutant strains of *P. solanacearum*. The resulting mutants retained pathogenicity but were reduced in virulence, suggesting that cell wall-degrading enzymes may not be essential for wilting, but do contribute to disease development (Roberts *et al.* 1988; Schell *et al.* 1988).

We recently described an extracellular protein from *P. solanacearum* that induces a rapid browning response in tissue culture calli of incompatible, but not of compatible lines of the cultivated potato diploid *Solanum phureja* Juz. and Buk. (Huang *et al.* 1989). Based on the strong correlation between the rapid browning response to bacteria in tissue culture and the HR in the corresponding whole plant lines, we hypothesized that this protein might play a role in the HR of nonhost plants to *P. solanacearum*. In this study, we present evidence that this protein is a PG. We have cloned the structural gene for the major extracellular PG from strain K60 and studied its expression in culture and in the plant. In addition, we isolated a region of DNA that is required for PG expression and may encode a positive regulatory factor.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids and their relevant characteristics are given in Table 1.

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Growth of bacterial strains. Strains of *P. solanacearum* were cultured at 28° C in CPG broth (Hendrick and Sequeira 1984) or on plates containing CPG plus 1.8% agar and 0.05% 2,3,5-triphenyltetrazolium chloride (TZC medium) (Kelman 1954). Boucher's minimal medium (BMM) (Boucher *et al.* 1985) was used when a defined medium was required. Strains of *Escherichia coli* were grown at 37° C in Luria-Bertani medium (Miller 1972). The following antibiotics were added as required: ampicillin, 50 μg/ml; chloramphenicol, 25 μg/ml; kanamycin, 50 μg/ml; nalidixic acid, 75 μg/ml; and tetracycline, 25 μg/ml.

Chemicals and enzymes. Restriction enzymes and T4 DNA ligase were purchased from Promega Corporation (Madison, WI). Calf intestinal phosphatase and T1 RNase were from Boehringer Mannheim Biochemicals (Indianapolis, IN). Nick translation kits were purchased from Bethesda Research Laboratories (Gaithersburg, MD) and used according to the supplier's directions. Radiochemicals were purchased from Amersham (Arlington Heights, IL). Electrophoresis chemicals and nylon transfer membranes were from Bio-Rad Laboratories (Richmond, CA). Growth medium components were from Difco Laboratories (Detroit, MI). Other chemicals were from Sigma Chemical Co. (St Louis, MO).

DNA manipulations. Isolation of plasmid DNA, restriction mapping, subcloning, transformation of *E. coli* strains, and Southern blot hybridization were performed as described previously (Maniatis *et al.* 1982). Strains of *P. solanacearum* were transformed by electroporation with a Genezapper apparatus from International Biotechnologies, Inc., (New Haven, CT) at a capacitance of 21 microfarads and an electric field of 10 kV/cm. Mid log phase cells were made competent for electroporation by washing them successively in 1, 0.5, 0.1, and 0.01 volumes of 10% glycerol. Competent cells were stored at −80° C.

Transposon and marker exchange mutagenesis. Mutagenesis with the transposon Tn*5* was performed as described previously (Xu *et al.* 1988). A modification of the method of Stachel *et al.* (1985) was used to mutagenize plasmids with a Tn*3* construct containing a β-glucuronidase (*gus*) reporter gene (Bonas *et al.* 1989). Plasmids were also mutagenized with transposon Tn*5*ORF*lac*, which generates translational fusions to a *lac* reporter gene (Krebs and Reznikoff 1988). Marker exchange mutagenesis of wild-type strains of *P. solanacearum* with DNA containing Tn*3gus* or Tn*5*ORF*lac* insertions was conducted as described previously (Xu *et al.* 1988), except that mutated DNAs were introduced into wild-type strains by electroporation.

Plasmid construction. Cosmid pPG1 was isolated from a genomic library of strain K60 in cosmid pLAFR3 carried in *E. coli* DH5 (Xu *et al.* 1988) during a screening of 1,500 clones for production of pectin-degrading enzymes on a pectinase-detection medium (Reverchon *et al.* 1985). The Tn*5*-containing DNA fragments from mutant strains were cloned after digesting total genomic DNA from the mutants with *Eco*RI. The digested DNA was electrophoresed in 0.5% agarose, and a gel slice containing the appropriate size range of fragments was excised. DNA was removed from the gel with GeneClean (Bio-101, Inc., La Jolla, CA) and ligated to *Eco*RI-digested pUC18 DNA. The resulting chimeric plasmids were transformed into DH5 and kanamycin-resistant strains were selected. The inserts from these plasmids were used to probe colony blots of the K60 or B1 genomic libraries to identify cosmid clones with homology to the Tn*5*-containing fragment. Plasmid pMB11ΔSal was a generous gift from Mark Schell, University of Georgia, Athens.

Screening for plant response. Plant pathogenicity assays were performed on eggplant (*S. melongena* L. cv. Black Beauty) seedlings as previously described (Xu *et al.* 1988). Strains were tested for the HR on tobacco (*Nicotiana tabacum* L. cv. Bottom Special) and cucumber (*Cucumis sativus* L. cv. Wisconsin SMR18) leaves as described by Klement *et al.* (1964). The rapid browning response of *S. phureja* tissue culture calli and the viability of tissue culture cells by staining with fluorescein diacetate were assayed as described previously (Huang *et al.* 1989). Populations of bacteria in tobacco leaf tissue were measured as described previously (Sequeira and Hill 1974).

Protein purification and enzyme assays. Initially, assays for PG activity were performed with an approximately 60-kDa protein purified from culture supernatants as described previously (Huang *et al.* 1989). An abbreviated protein purification method was developed as follows: the supernatant from a bacterial culture at $OD_{600\ nm} = 0.3$–0.6 was passed through a 4 × 1 cm DEAE-cellulose DE52 (Whatman, Clifton, NJ) column with 20 mM KH_2PO_4, 1 mM

Table 1. Bacterial strains and plasmids used in this study

Designation	Relevant characteristics[a]	Source or reference
Escherichia coli		
DH5	*recA1 endA1*	Hanahan 1983
C2110	Nal^r *polA*	Stachel *et al.* 1985
HB101	*recA13*	Maniatis *et al.* 1982
Pseudomonas solanacearum		
K60	Wild-type race 1, biotype 1	Kelman 1954
B1	Spontaneous nonpathogenic mutant of K60	Kelman 1954
K18	K60::Tn*5* PG$^-$	This study
K19	K60::Tn*5* PG$^-$	This study
K60/1	K60 *pehA*::Tn*3gus*	This study
B5	B1::Tn*5* Pg$^-$	This study
B1/1	B1 *pehA*::Tn*3gus*	This study
Plasmids		
pBluescript KS+	Ap^r	Stratagene, Inc.[b]
pLAFR3	Tc^r	Staskawicz *et al.* 1986
pHO*gus*	Tn*3gus* Km^r Ap^r	Bonas *et al.* 1989
pRZ620	Tn*5*ORF*lac* Tc^r Col^r	Krebs and Reznikoff 1988
pUC18	Ap^r	Yanisch-Perron *et al.* 1985
pPG2	PehA$^+$ Tc^r	This study
pPG1/1	*pehA*::Tn*3gus* Tc^r Km^r	This study
pPG3	PehA$^+$ Ap^r	This study
pPG1*lac5*	*pehA*::Tn*5lac* Tc^r	This study
pKH19	PehR$^+$ Tc^r	This study
pK19	pUC18 with a 10.2-kilobase *Eco*RI fragment from pK18. Ap^r Km^r	This study

[a] Nal, nalidixic acid; Ap, ampicillin; Tc, tetracycline; Km, kanamycin; Col, colicin; and ^{r}, resistant.
[b] La Jolla, CA.

EDTA, pH 7.0, buffer at a flow rate of about 15 ml/hr; the column was washed with one column volume of the same buffer. The total eluate was concentrated 50- to 150-fold in an Amicon concentrator provided with a YM30 membrane (Amicon, Danvers, MA). PG activity was assayed by means of the McFeeters' reducing sugar assay (McFeeters 1980) and normalized to galacturonic acid equivalents. Assays for changes in viscosity of a pectate solution and for pectate lyase activity were performed as described previously (Keen *et al.* 1984; Sherwood and Kelman 1964). Pectolytic activity in isoelectric focusing gels was determined by the thin-layer substrate overlay method of Ried and Collmer (1985). We measured production of β-glucuronidase activity by Tn*3gus* mutant strains by fluorometry (Jefferson 1987) of either culture supernatants (for secreted activity) or sonicated pellets (for intracellular activity). Production of β-galactosidase was measured as described by Miller (1972).

Measurement of *in planta* PG levels. To measure the total production of bacterial PG activity *in planta*, we used a syringe to infiltrate fully expanded leaves of tobacco cultivar Bottom Special with bacteria that had been grown in BMM plus 0.2% glycerol to mid exponential phase, centrifuged at room temperature, and resuspended in sterile distilled water to $OD_{600\ nm} = 0.5$ (approximately 5×10^8 colony-forming units per milliliter). The plants were placed in a growth chamber for 18 hr at 28° C. Intercellular fluids containing extracellular PG were extracted from excised infiltrated areas by repeated vacuum infiltration with water followed by centrifugation (Klement 1965). The enzyme was purified from these fluids as described above. The total number of bacteria present per gram of infiltrated leaf area was determined by dilution plating from homogenized infiltrated tissue as described previously (Sequeira and Hill 1974).

RESULTS

Identification of the protein responsible for callus rapid browning. Since the browning factor protein described by Huang *et al.* (1989) induced maceration of potato cells as well as the rapid browning response, a sample of previously purified browning factor protein from strain B1 was tested for pectolytic activity. The sample had no pectate lyase activity, but did have high endopolygalacturonase activity, as determined by the McFeeters' reducing sugar assay and viscometry. Strain K60 produces at least two extracellular PGs; one, with a pI of 9.0, is responsible for about 90% of the PG activity produced by the strain in culture. A second PG with a pI of 8.0 makes up the remaining PG activity produced in culture (Fig. 1, lane c).

Cloning of the PG gene. Four cosmid clones encoding PG activity were identified by screening 1,500 clones from a *P. solanacearum* K60 genomic library in *E. coli* DH5. Restriction mapping and Southern blot analysis indicated that the four cosmids contained overlapping DNA inserts; one cosmid, designated pPG1, was chosen for further study. Subcloning and mutagenesis with the Tn*3gus* transposon-reporter gene construct allowed us to determine the apparent limits of the structural gene and the direction of transcription (Fig. 2). The intracellular protein extracted from *E. coli* DH5 carrying subclone pPG3 was tested for PG activity by means of an activity overlay either following isoelectric focusing or after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. We found that pPG3 encoded production of a PG with a molecular size of about 58 kDa and a pI of 9.0 (Fig. 1). This protein had browning activity on tissue culture callus, and its physical characteristics agree with those of an extracellular PG isolated from K60, with those of the previously described rapid browning factor from strain B1, and also with those of the *P. solanacearum* PG described by Schell and co-workers (1988). We designated this gene *pehA* (*p*ectic *e*nzyme-*h*ydrolase) in keeping with the convention for naming pectic enzymes (proposed nomenclature for genes of degradative enzyme production, European Molecular Biology Organization Workshop on Soft-Rot Erwiniae, July 23–27, 1984, Marseille-Luminy, France). Southern blot hybridization experiments showed that pPG4, carrying part of the *pehA* locus, has strong homology to the insert of pMB11ΔSal,

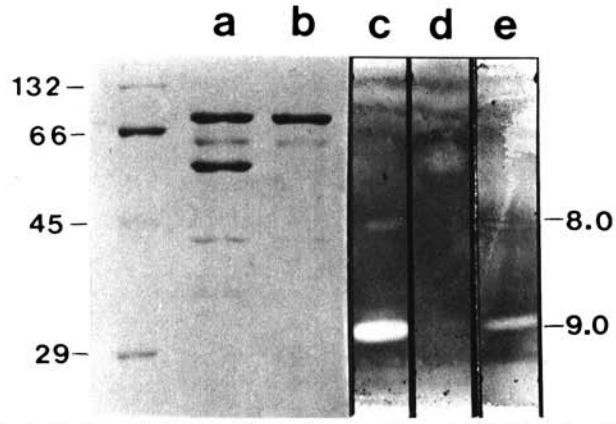

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel of total extracellular protein (left) and pI 7.0–10.0 isoelectric focusing gel pectolytic activity overlay of purified extracellular (strains of *Pseudomonas solanacearum*) or intracellular (strain of *Escherichia coli*) polygalacturonase (right). Lanes a and c, *P. solanacearum* K60; lanes b and d, site-specific marker exchange mutant K60/1; and lane e, *E. coli* (pPG3).

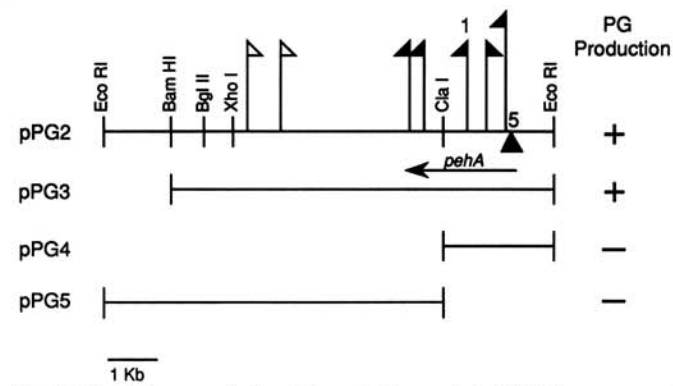

Fig. 2. Physical maps of plasmids containing *pehA*. Solid flags represent Tn*3gus* insertions that result in a polygalacturonase-minus (PG^-) phenotype when the mutagenized plasmid is carried in *Escherichia coli*; open flags represent insertions that do not affect PG production. The orientation of the flags indicates the orientation of the inserted *gus* reporter gene. The Tn*3gus* insertion used to generate marker exchange mutants K60/1 and B1/1 is labeled 1. The triangle labeled 5 represents the insertion point of Tn*5*ORF*lac*. The arrow indicates the probable location and transcriptional direction of the *pehA* gene.

which encodes the PG (PglA) from *P. solanacearum* AW1 described by Schell and co-workers (1988) (data not shown).

Homology of pPG1 to the Vir2 *hrp* cluster. We used pPG1 to probe Southern blots of several *P. solanacearum* clones containing DNA required for plant-bacterial interactions (*hrp*, *eps*, or *vir* loci). The insert of pPG1 had strong homology to the *hrp*-encoding cosmid pL810 (data not shown). pL810 overlaps with another *hrp*-encoding cosmid, pL1506 (Xu *et al.* 1988). pL1506 in turn has homology to pVir2 (Douglas Cook, unpublished data), a 25-kb region of *P. solanacearum* DNA containing a cluster of *hrp* loci described by Boucher and co-workers (1987) (Fig. 3).

Site-specific mutagenesis of the PG gene. Five transposon Tn*3gus* insertions in the *pehA* locus (solid flags in Fig. 2) were marker-exchanged into the genomes of both strain K60 and the spontaneous mutant strain B1 and confirmed by Southern blot analysis (data not shown). The resulting tetracycline-sensitive and kanamycin- and ampicillin-resistant mutants no longer produced the 58-kDa PG with a pI of 9.0 *in vitro* as indicated by isoelectric focusing gels and sodium dodecyl sulfate-polyacrylamide gels of total extracellular protein (Fig. 1, lanes b and d). A representative marker exchange mutant, K60/1, retained pathogenicity on tobacco (15 of 15 plants tested were wilted) and eggplant (30 of 30 seedlings tested were killed) and was still able to induce an HR on cucumber seedling cotyledons; in short, it behaved as expected for the wild-type parental strain, K60. The equivalent mutant of strain B1, B1/1, induced an HR on tobacco leaves that was indistinguishable from that induced by B1. Both K60/1 and B1/1 were unable to induce the rapid browning response on potato callus, confirming that *pehA* encodes the browning factor described by Huang and co-workers (1989).

Regulation of PG production. We measured relative levels of total PG produced by strain K60 cells grown in rich medium (CPG), minimal medium (BMM), minimal medium plus 5% tobacco leaf intercellular fluids, and in tobacco leaves. Although PG was produced at a low basal level in rich media (5.5 μmol of galacturonic acid equivalents per minute), higher levels of PG activity (21.3 μmol/min) were produced in minimal medium. When 5% tobacco leaf intercellular fluid was added to minimal medium, a slight increase (104.2 μmol/min) in specific activity levels was observed. Cells grown directly in tobacco leaves produced substantially higher levels of activity (922.3 μmol/min) than those grown in minimal medium.

Identification of a second class of browning-minus mutants unaffected in the *pehA* structural gene. Mutants affected in positive regulators of PG production would be expected to have a PG^-, browning-minus phenotype. When 4,000 Tn*5*-carrying transconjugants, 3,000 from strain K60 and 1,000 from strain B1, were screened for a browning-minus phenotype based on the inability to kill and lyse *S. phureja* line C-3 cells, three distinct mutants were identified, one from B1 (B5) and two from K60 (K18 and K19). These mutants were also unable to induce the rapid browning response on *S. phureja* callus tissue, but retained the ability to induce the HR on incompatible lines of *S. phureja* plants and on cucumber seedlings. In addition, all three were affected in production of extracellular PG (see below). Mutant B5 retained the ability of its parent strain, B1, to induce the HR on tobacco leaves. Mutants K18 and K19, derived from virulent strain K60, were still virulent on tobacco and eggplant seedlings. All three mutants were prototrophic and grew as their parent strains did in tobacco leaves (data not shown). Southern hybridization analysis of total genomic DNA from each of the three mutants probed with ^{32}P-labeled Tn*5* DNA showed that each mutant contained a single Tn*5* insertion (Fig. 4).

Cloning the putative positive regulatory gene. We cloned the Tn*5*-containing *Eco*RI fragments from the three browning-minus mutants into pLAFR3, generating plasmids pB5, pK18, and pK19. Southern blot analysis of genomic DNA from parent strains B1 and K60 and mutants B5, K18, and K19 probed with pK19 showed that Tn*5* is inserted into a common DNA fragment in all three mutants (Fig.

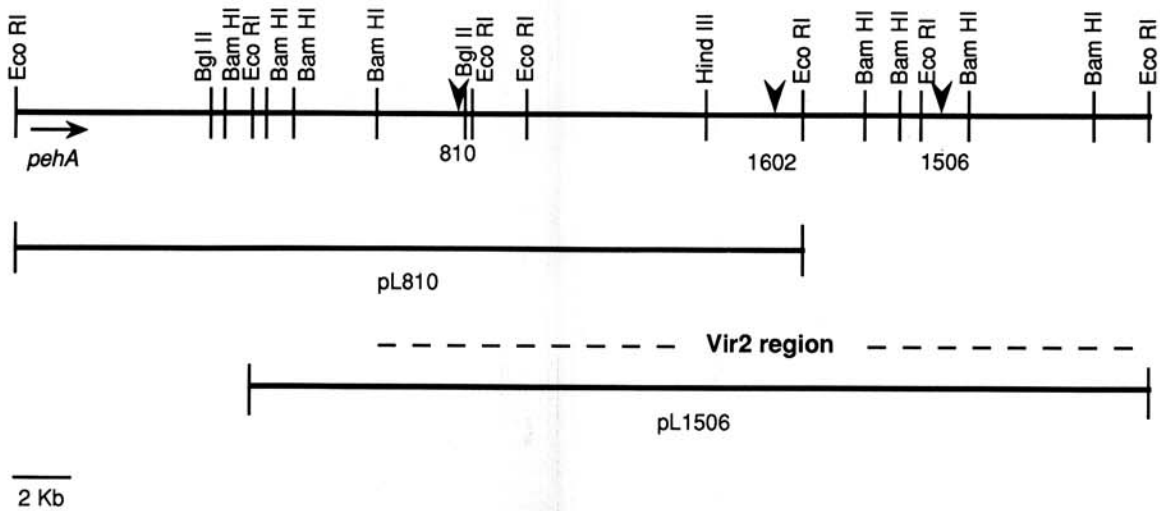

Fig. 3. Physical maps of cosmids pL810 and pL1506 from *Pseudomonas solanacearum* K60 (Xu *et al.* 1988) showing areas of overlap as determined by Southern blot analysis. Arrows indicate the sites of Tn*5* insertion in the original *hrp* mutants KD810, KD1602, and KD1506. Cosmid pVir2 (Boucher *et al.* 1988) from strain GMI1000 has strong homology to pL1506 right from the *Bam*HI site as indicated by the dashed line; however, since there is no restriction site homology between the two strains in this region, the boundary of homology is approximate.

4). The two Tn*5* insertions in strain K60 were in a 4.5-kb *Eco*RI fragment, but the B1 insertion was in a homologous 6-kb fragment because one of the *Eco*RI sites was absent in strain B1 (Fig. 4). When plasmid pB5 was marker-exchanged into the K60 genome, the resulting mutant, KB5, had the same phenotype as mutants K18 and K19. Since the Tn*5* insertions in pK18 and pK19 were in a homologous 4.5-kb *Eco*RI fragment, only pK19 was used as a probe to screen a K60 genomic library. pB5 was used as a probe to screen a B1 genomic library. Four cosmids were identified by this procedure, two (pK19A and pK19B) from K60 and two (pB5A and pB5B) from B1. Each cosmid was transformed into mutants K18, K19, and B5 by electroporation to determine its ability to restore induction of the rapid browning response on potato callus tissue. Each of the four cosmids was able to restore this phenotype in each of the three mutants. A subclone containing only the 4.5-kb *Eco*RI fragment from K60 (plasmid pKH19) also was able to complement all three mutant phenotypes back to the wild type, indicating that this fragment contained the complete complementation unit interrupted by the three Tn*5* insertions.

pKH19 was mutagenized with Tn*3gus* to determine the limits of the DNA region required for complementation of the mutant phenotypes. The Tn*3gus* insertions in pKG2, pKG5, pKG19, and pKG50 were mapped, and the level of β-glucuronidase expression conferred by each plasmid was measured in *P. solanacearum* to determine the direction of transcription of the gene(s) into which the transposon was inserted (Fig. 5). The four Tn*3gus*-containing plasmids were transformed into the three mutants to generate the corresponding *trans*-merodiploid strains, which were then assayed on callus tissues for induction of the rapid browning response. The ability to induce the browning response was not restored in the strains carrying pKG5, pKG19, and pKG50, suggesting that a single complementation unit was inactivated by the three transposon insertions. However, the mutants containing pKG2 caused the browning response, suggesting that in this plasmid Tn*3gus* is not inserted within the complementation unit. When the insertions in plasmids pKG5, pKG50, and pKG2 were marker-exchanged into the B1 chromosome, mutants BG5 and BG50 lost the ability to induce rapid browning, but BG2 still induced a wild-type rapid browning response. These results confirmed that in pKG2, Tn*3gus* is inserted outside of the complementation unit. The Tn*5* and Tn*3gus* insertions together define a region of at least 2.4 kb required for induction of the rapid browning response.

PG production by browning-minus mutants. We tested strains K18, K19, and B5 for production of extracellular PG. Mutants K18 and K19 produced no detectable PG activity, while mutant B5 produced about 60 times less PG than its parent strain B1 (Fig. 6). It is not surprising that the mutant derived from B1 should produce a higher level of PG than those derived from K60, since B1 itself produces about 25 times more PG than K60. A complemented strain, such as K18 carrying plasmid pKH19, produced about eight times the total PG activity of the parental strain K60 (Fig. 6).

We tested the effect of the mutation in K18 on expression of *pehA*, measured as β-galactosidase produced by a plasmid-borne *pehA*::Tn*5lac* fusion, pPG1*lac*5. Strain K60 carrying this construct produced 1,709 units of β-galactosidase activity (measured as nanomoles of *o*-nitrophenyl β-D-galactopyranoside hydrolyzed per minute per milligram dry weight of bacteria). However, strain K18 carrying the same construct produced only 152 units of β-galactosidase activity.

Southern hybridization experiments indicated that there is no detectable homology between the *pehA* structural gene described previously and the pKH19 region described above (data not shown). *E. coli* cells carrying pKH19 produced no detectable PG activity. These results suggest that although the DNA insert of pKH19 is required for expression of PG, it does not itself encode a structural gene for the enzyme. Lysates from strains K18 and K19 contain no detectable PG activity, whereas lysates from strain K60 contained 160 units of PG activity per milligram dry weight of bacteria (PG activity expressed as micromoles of galacturonic acid equivalents released per minute). It is therefore unlikely that these mutants are deficient in secretion of PG. We named this mutation *pehR* since it

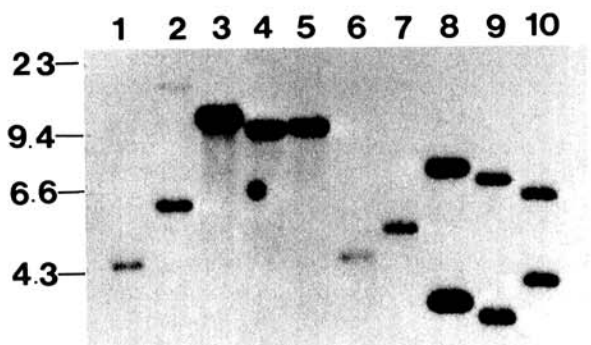

Fig. 4. Southern hybridization analysis of total chromosomal DNA from strains K60, B1, B5, K18, and K19 of *Pseudomonas solanacearum*. Total genomic DNA was digested with *Eco*RI (lanes 1–5) and *Eco*RI and *Bam*HI (lanes 6–10) and probed with pK19 DNA. Lanes 1 and 6 contain K60; lanes 2 and 7, B1; lanes 3 and 8, B5; lanes 4 and 9, K18; and lanes 5 and 10, K19. The hybridization pattern indicates that the sequences flanking the Tn*5* insertions in the three mutants are homologous.

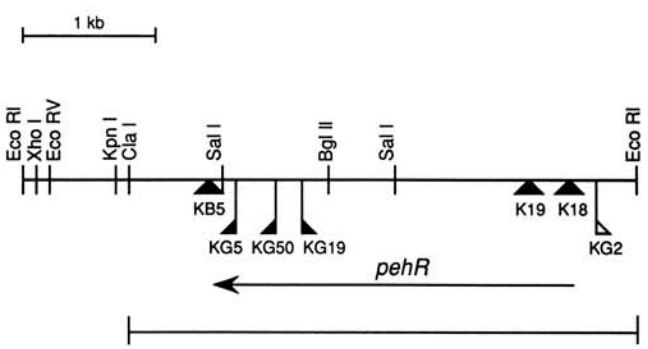

Fig. 5. Physical map of plasmid pKH19. Solid triangles indicate sites of Tn*5* insertion in the original polygalacturonase-minus mutants; flags indicate sites of Tn*3gus* insertions. Solid flags represent insertions in the *pehR* complementation unit, and the open flag represents an insertion outside the complementation unit. The orientation of the flags indicates the orientation of the inserted reporter gene. The arrow shows the probable location and transcriptional direction of the *pehR* gene(s).

appears to have a regulatory effect on PG production.

DISCUSSION

We describe here the cloning of the gene for a *P. solanacearum* protein that induces a rapid browning response in *S. phureja* tissue culture callus (Huang *et al.* 1989). We have determined that this protein is an extracellular endopolygalacturonase of about 58 kDa with a pI of 9.0. The gene encoding this PG, called *pehA*, is located about 15 kb from one end of the Vir2 region (Fig. 3), a cluster of previously identified *hrp* loci in *P. solanacearum* (Boucher *et al.* 1987; Xu *et al.* 1988). The location of *pehA* supports the idea that genes involved in plant-bacterial interactions are physically clustered in the *P. solanacearum* genome. When the mutagenized PG gene was marker-exchanged into the wild-type *P. solanacearum* chromosome to generate a strain unable to produce the PG with a pI of 9.0, the resulting mutant also did not induce the rapid browning response in *S. phureja* tissue culture calli. Furthermore, *E. coli* cells carrying the cloned *pehA* gene produced a protein that elicited the rapid browning response. Thus, we conclude that this PG is necessary for induction of the rapid browning response.

In a previous article (Huang *et al.* 1989), we suggested that the factor responsible for the rapid browning response of potato callus tissues and death of suspension-cultured cells might be specifically required for HR induction. This was based on the strong correlation between bacterial induction of the browning response on callus clones and the HR obtained in the corresponding intact plant lines. However, since the PG^- marker exchange mutant was still able to induce an HR on incompatible plant hosts, it is evident that the inability to produce the PG with a pI of 9.0 and the rapid browning response does not affect induction of the HR in intact plants. Furthermore, the mutant was still pathogenic on compatible hosts. We therefore conclude that this PG is not required for either the HR or pathogenicity and that callus browning is probably not analogous to the HR. The nature of the browning response of tissue culture callus to bacteria or to purified PG remains unknown and may be specific to tissue culture. Some products of pectolytic activity are known to be toxic to plant cells and others elicit various specific responses from plants (reviewed in Ryan 1987). For example, Bruce and West (1982) found that products of a fungal PG induce production of the phytoalexin casbene in castor bean plants. By analogy, we speculate that PG may release products of cell wall degradation that act as specific elicitors of browning in callus tissues but that are not required for HR induction in the intact plant. Alternatively, different products with separate activities may be released by the action of PG in whole plants as compared with tissue culture.

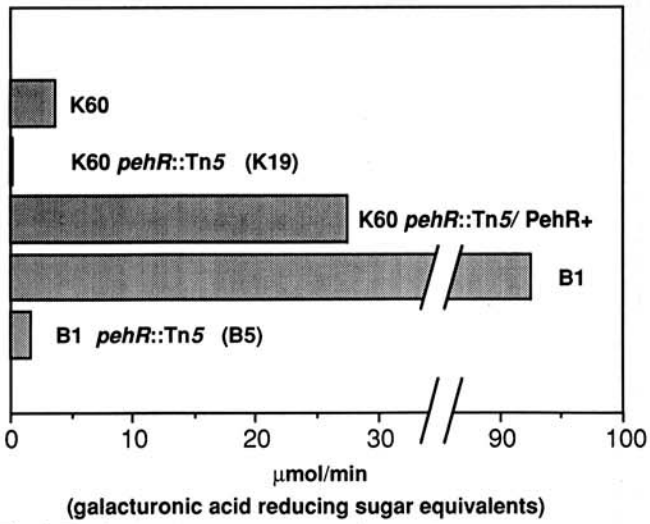

Fig. 6. Total polygalacturonase production by wild-type and *pehR* mutant strains of *Pseudomonas solanacearum*. Activity is expressed as micromoles of galacturonic acid equivalents per minute.

We believe that the cloned PG gene, *pehA*, is the K60 homologue of *pglA*, the gene described by Schell *et al.* (1988) from strain AW1 of *P. solanacearum*. The *pehA* gene from strain K60 had strong DNA homology to *pglA*, and the two enzymes encoded by these genes had similar physical characteristics. Mark Schell and co-workers found that a PG^- mutant of strain AW1 was slightly reduced in virulence as determined by a tomato stem inoculation assay. The PG^- mutant of K60, however, did not appear to be affected in pathogenicity. The eggplant and tobacco seedlings inoculated in our pathogenicity assays may be more susceptible than the tomato plants used by Mark Schell and co-workers and therefore might not detect a subtle reduction in virulence. We should point out that neither marker exchange mutant is completely PG^-, since *P. solanacearum* produces a minor PG with a pI of 8.0 in addition to the major PG with a pI of 9.0 discussed here. Although the second PG comprises only about 10% of the total PG activity produced by the bacterium in culture, the relative amounts of the two enzymes produced in the plant are not known. A double mutant unable to make both PGs is required before we can conclusively define the necessity of PG for disease development.

We found that basal level production of total PG was very low when *P. solanacearum* K60 was grown in rich media; when it was grown in a defined minimal medium, production of PG increased 10-fold. This may reflect catabolite repression, or there may be specific induction by a nutrient-poor medium, such as reported by Huynh *et al.* (1989) for a *hrp* gene of *P. syringae* pv. *glycinea* (Coerper) Young *et al.* There was a further increase (about fivefold) in PG activity when the bacteria were grown in minimal medium supplemented with intercellular fluids from tobacco leaves and a greater than 100-fold increase when bacteria grew directly in tobacco leaves. This suggests that in addition to a small induction caused by nutrient stress or from relief of catabolite repression in minimal media, there is an additional factor in plant tissue that induces substantial increases in PG production. Again, we were unable to distinguish between the two isozymes of PG in performing these induction studies; it is possible that they are differentially induced.

We reasoned that if PG were subject to positive regulation, a population of Tn*5*-generated PG^- mutants would include some PG^-, browning-minus strains affected in positive regulatory element(s). We isolated three Tn*5* mu-

tants that are deficient in their ability to induce rapid browning of *S. phureja* callus tissues. Although two of these mutants were derived from a wild-type strain and one from a spontaneous, nonpathogenic mutant strain, genetic analysis showed that Tn*5* was inserted in the same DNA region in all three mutants. Surprisingly, an *Eco*RI site present in the K60 DNA was absent from the corresponding fragment in B1; although the homologous fragments from the two strains were functionally interchangeable for their effect on callus browning and PG production, this restriction fragment length polymorphism may be representative of the genetic rearrangements observed in spontaneous, nonpathogenic mutants of *P. solanacearum* (Douglas Cook, unpublished observations). Since the corresponding wild-type cosmids from the two parental strains were able to complement all three mutations, these mutations probably interrupt a single complementation unit. We called this unit *pehR*. Although the mutated DNA region had no homolgy to the PG structural gene described above and did not enable production of PG by strains of *E. coli*, the three mutants produced greatly reduced or undetectable amounts of PG. Interestingly, when the mutant strains were complemented with the wild-type sequence, the resulting *trans*-merodiploid strains produced about eight times the wild-type level of PG. Since this complemented strain carried the wild-type *pehR* sequence on the low copy number plasmid pLAFR3 (three to five copies per cell; Figurski *et al.* 1979) and overproduced PG about eightfold relative to the wild-type strain, gene copy number is probably responsible for the overproduction of PG. *pehR* apparently acts at the transcriptional level on *pehA*, since expression of a *pehA*::Tn*5lac* reporter gene construct was reduced about 10-fold in a *pehR* mutant background. These results suggest that *pehR* encodes a positive *trans*-acting function involved in PG production. Work is currently underway to further characterize the gene and its mutant phenotype.

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