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Polygalacturonase Is a Virulence Factor in *Agrobacterium tumefaciens* Biovar 3

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Agrobacterium tumefaciens **biovar 3 causes both crown gall and root decay of grapes. All biovar 3 strains, regardless of their tumorigenicity, produce in culture a single polygalacturonase with a pI around 4.5. *A. tumefaciens* biovar 3 strain CG49 was mutagenized with Tn*5* by using pSUP2021 as a suicide vector. A mutant strain, CG50, lacking polygalacturonase activity was isolated. The mutation was due to a single Tn*5* insertion in an 8.5-kb *Eco*RI fragment that also contained the polygalacturonase structural gene. The polygalacturonase-encoding *pehA* gene was cloned in *Escherichia coli* by using the plasmid pBluescript as a vector. Activity-stained isoelectric focusing gel analysis demonstrated that *E. coli* cells harboring the $pehA^+$ recombinant plasmid pCPP2067 produced a polygalacturonase in culture with the same pI as the enzyme produced by CG49. The *pehA* gene was localized within a 2.5-kb *Hin*dIII-*Sal*I fragment. This fragment was used as a probe in Southern hybridization analysis and showed that no closely related genes are present in *A. tumefaciens* biovars 1 or 2, *Rhizobium leguminosarum*, or *Bradyrhizobium japonicum*. The polygalacturonase mutant was unable to induce root decay in grapes (*Vitis vinifera* cv. Chardonnay) and was substantially less tumorigenic than the wild type in grape stems when low levels of inoculum were used, although both strains were equally tumorigenic in potato disc assays. The results indicate that polygalacturonase is a virulence factor in both the root decay and crown gall incited in grapes by *A. tumefaciens* biovar 3.**

Agrobacterium tumefaciens biovar 3 (= *Agrobacterium vitis* [20]) is the causal agent of crown gall of grapevine, an important disease in all major viticultural areas (3, 26). Although it is tumorigenic in other hosts (20), biovar 3 shows a great degree of specificity for grapevine (4, 8), in which it survives systemically and can be disseminated in propagation material (7). In addition to crown gall, biovar 3 causes a root decay that is specific to grape roots (6).

All *A. tumefaciens* biovar 3 strains studied, regardless of tumorigenicity, produce a single, extracellular polygalacturonase, as indicated by activity-stained isoelectric focusing gels, whereas other biovars produce neither root decay of grape seedlings nor extracellular polygalacturonase in culture (19). Polygalacturonase activity can be recovered from root lesions infected with biovar 3 (19), and the maceration observed in these lesions is typical of the symptoms caused by pectolytic pathogens (13). These observations support the hypothesis that polygalacturonase is a disease determinant in *A. tumefaciens* biovar 3. To test the postulated involvement of polygalacturonase in root decay and to explore the possible role of polygalacturonase in tumorigenicity, we isolated a polygalacturonase-deficient mutant of *A. tumefaciens* biovar 3 and then cloned the polygalacturonase-encoding *pehA* (pectic enzyme hydrolase) gene.

A preliminary report by Ophel et al. (21) of the cloning of a polygalacturonase gene from *A. tumefaciens* biovar 3 has appeared, and we have published a preliminary account of part of this work (12). This is the first report of the mutagenesis and cloning of a functional pectic enzyme gene in a member of the family *Rhizobiaceae*.

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MATERIALS AND METHODS

Bacterial strains and plasmids. Strains of *A. tumefaciens* and *Escherichia coli* are listed in Table 1. The plasmid pSUP2021 (25) was used for Tn*5* mutagenesis; the plasmids pBluescript KS(−) and pBluescript SK(−) (Stratagene, La Jolla, Calif.) were used for cloning in *E. coli*.

Media and culture conditions. *A. tumefaciens* was grown at 28°C in either Kado medium 523 (18) or yeast-mannitol medium (16). *E. coli* strains were grown at 37°C in L broth (23). *A. tumefaciens* transconjugants were selected on AB minimal medium (11).

Transposon mutagenesis. A spontaneous Rif^r derivative of *A. tumefaciens* biovar 3 strain CG49 was selected by plating bacteria on rifampin gradients plates (0 to 100 μg/ml) for three successive cycles. The Rif^r strain, designated CG49R, was indistinguishable from CG49 in its growth in various media and its ability to cause root decay. *E. coli* SM10 donor cells, harboring the plasmid pSUP2021, and *A. tumefaciens* biovar 3 strain CG49R recipient cells were grown separately on plates. Bacterial cells were then resuspended in 10 mM potassium phosphate, pH 7.0, to an optical density at 600 nm of 1.0 (about 10^8 CFU ml^{-1}) and mixed at a ratio of 1:5 (donor to recipient). A 125-μl portion of the mating mixture was spread onto a nutrient agar plate and incubated at 37°C for 30 min and then shifted to 28°C overnight. Cells were washed in 2 ml of 0.8% NaCl and spread on AB minimal medium containing kanamycin (50 μg/ml) and rifampin (100 μg/ml).

Enzyme activity assays. Polygalacturonase activity in bacterial colonies was assayed by transferring the cells from an approximately 5-mm^2 area of each colony to a petri dish containing 5 ml of a medium consisting of 0.1% polygalacturonate (Pfaltz and Bauer, Inc), 50 mM potassium acetate (pH 4.5), and 1% agarose. Plates containing bacterial colonies spotted on the thin layer of polygalacturonate-agarose

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Reference or source
Strains		
A. tumefaciens biovar 3		
CG49	Wild type, tumorigenic	T. J. Burr
CG49R	Rif[r] derivative of CG49	This work
CG50	*pehA*::Tn*5* mutant of CG49R	This work
A. tumefaciens biovar 1 strain C58	Wild type, tumorigenic	R. S. Dickey
A. tumefaciens biovar 2 strain R-3	Wild type, tumorigenic	R. S. Dickey
R. leguminosarum	Wild-type 128C53	T. A. LaRue
B. japonicum	Wild-type lab strain	T. A. LaRue
E. coli		
SM10	C600, *thi thr leu recA* Muc$^+$ chromosomally integrated	25
DH10B	Sm^r, high efficiency in electroporation	9
Plasmids		
pSUP2021	Cm^r Km^r Tc^r	25
pBluescript	Amp^r	Stratagene
pCPP2067	Amp^r; 5.8-kb insert containing *pehA* gene, in pBluescript SK(−)	This work
pCPP2068	Amp^r; 2.8-kb *Hin*dIII-*Pst*I insert containing *pehA* gene, in pBluescript KS(−)	This work

were incubated at 28°C overnight and developed with a 1% (wt/vol) solution of hexadecyltrimethylammonium bromide (Sigma). A clear halo in a turbid background developed around colonies producing polygalacturonase. Polygalacturonase was also assayed with the Nelson's reducing sugar assay, although this assay was substantially less sensitive than the thin-layer substrate assay (14). Activity-stained isoelectric focusing gel analysis was performed as previously described (19).

General DNA manipulations. Plasmid DNA was isolated and manipulated by following standard techniques (23). Chromosomal DNA was isolated by the method of Silhavy et al. (24). DNA-modifying enzymes were purchased from Bethesda Research Laboratories or Promega. *E. coli* and *A. tumefaciens* cells were transformed by electroporation according to the method of Dower et al. (15). Nested deletions were made by using the Erase-A-Base Kit (Promega).

Cloning of the *pehA* gene from CG49. DNA from *A. tumefaciens* CG49 was partially digested with *Sau*3A and fractionated by electrophoresis on a 0.7% agarose gel. A gel slice containing DNA fragments 5 to 9 kb in size was excised. The DNA fragments were extracted by electroelution in an Elutrap apparatus (Schleicher & Schuell) and then ligated in the *Bam*HI site of pBluescript SK(−). Transformant DH10B colonies were screened for polygalacturonase activity by transferring colonies grown on Luria broth plates containing ampicillin (200 μg/ml) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (0.1 mg/ml; to confirm that colonies contained plasmids with inserts) to a plate containing a thin layer of polygalacturonate agarose (described above).

Southern blot analysis. Restriction enzyme-digested DNA fragments were separated by agarose gel electrophoresis, blotted onto Immobilon-N membranes (Millipore), and hybridized by following the manufacturer's instructions. Probe DNA was labeled by the method of Feinburg and Vogelstein (17) and used at a concentration of 1 ng/ml.

Pathogenicity assays. Assays to measure root decay activity were done as previously described (5, 6). Quantitative assays for levels of tumorigenicity were conducted on potato discs as described by Pueppke and Benny (22) and on woody stems of grapes (*Vitis vinifera* cv. Chardonnay) in the greenhouse. Dormant grape cuttings were rooted and then planted individually ca. 1 month prior to inoculation. Wounds in the woody stems were made by drilling four holes (about 2 mm in diameter), equidistant from each other, completely through the stems with an electric drill. Inoculum was grown for 48 h at 28°C on potato dextrose agar plates, adjusted to a titer of 10^8 CFU/ml with a spectrophotometer, and serially diluted. Twenty microliters of the different concentrations of inoculum was injected into each of four wound sites and then wrapped with a layer of Parafilm. Numbers and sizes of tumors were recorded after 2 months.

RESULTS

Construction of a Peh$^-$ mutant by Tn*5* mutagenesis. *A. tumefaciens* biovar 3 strain CG49R was mutagenized with Tn*5* by using the suicide vector pSUP2021 (25). Km^r Rif^r transconjugant colonies were obtained at a frequency of 10^{-6} per donor cell. Approximately 8,000 mutants were grown on Kado 523 agar medium and subsequently tested for polygalacturonase production by transfer to thin-layer polygalacturonate-agarose plates. The assays were performed this way because the low pH that was optimum for enzyme activity was inhibitory to bacterial growth. Only one polygalacturonase-deficient mutant, designated CG50, was isolated.

Characterization of Peh$^-$ mutant CG50. The polygalacturonase produced by *A. tumefaciens* biovar 3 is largely extracellular (19). Therefore, the nonpectolytic phenotype of CG50 could be explained by mutation in a gene involved in secretion rather than in enzyme production. To test this possibility, polygalacturonase activity was assayed in both the extracellular fraction and in sonicated cells from CG50 cultured in 523 medium. No traces of activity were detected in either fraction.

Southern blot hybridization was used to detect the presence of Tn*5* sequences in CG50 DNA. As shown in Fig. 1, a single hybridizing band, with a mobility corresponding to 14.0 kb, appeared when using DNA from CG50, whereas no bands were detected with wild-type DNA. The chromosomal DNA was digested with *Eco*RI because this enzyme does not cut within Tn*5*. The result indicates that a single transposition event took place in CG50.

To further confirm that the Peh$^-$ phenotype in CG50 was due to the Tn*5* insertion, a 7.0-kb *Cla*I fragment from CG50 was cloned in the vector pBluescript SK(−). The recombinant plasmid, containing Tn*5* and 1.6 kb of flanking sequences, was marker exchanged into the CG49 genome. All Km^r bacteria analyzed were also defective in polygalacturonase production.

Mutant CG50 was also found to be Rif^s. Although rifampin was used (in addition to minimal medium) to counterselect the donor during Tn*5* mutagenesis of CG49R, the resulting Km^r mutants were subsequently maintained in the absence of rifampin selection, and resistance to rifampin was spon-

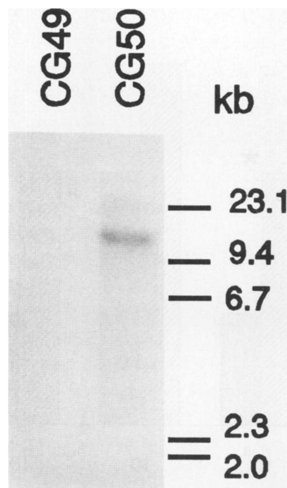

FIG. 1. Detection of Tn*5* sequences in *Eco*RI-digested chromosomal DNA of Peh⁻ *A. tumefaciens* biovar 3 mutant CG50. pSUP2021 DNA, which contains Tn*5*, was labeled with [^{32}P]dCTP and used as a probe. Parental strain CG49 was probed in the first lane.

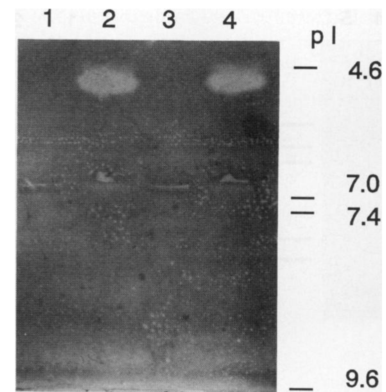

FIG. 3. Activity-stained isoelectric focusing gel analysis of polygalacturonase production by *A. tumefaciens* biovar 3 wild-type and mutant strains and by a *pehA*$^+$ *E. coli* strain. Supernatants of *A. tumefaciens* cultures and sonicated extracts of *E. coli* cells were resolved by ultrathin-layer isoelectric focusing and then activity stained with an ultrathin pectate-agarose overlay. Lanes: 1, *A. tumefaciens* biovar 3 strain CG50; 2, *A. tumefaciens* biovar 3 strain CG49; 3, *E. coli* DH10B; 4, *E. coli* DH10B containing *pehA*$^+$ plasmid pCPP2067. The activity-stained overlay contained polygalacturonate buffered with potassium acetate at pH 4.5 to optimize polygalacturonase activity.

taneously lost. Strain CG50 still cross-reacted with biovar-specific antibodies (3), and it grew at rates equivalent to those of the wild type and produced characteristic colonies on Kado medium 523, yeast-mannitol medium, and AB minimal medium.

Molecular cloning of the *pehA* gene. A CG49 DNA library was constructed in pBluescript SK(−) and used to transform *E. coli* DH10B cells. Approximately 3,000 white Amp^r colonies were tested for polygalacturonase activity by using the thin-layer polygalacturonate-agarose plates described above. One colony that produced a low but detectable level of enzyme was identified. The corresponding recombinant plasmid, designated pCPP2067, contained a 5.8-kb DNA insert (Fig. 2). A set of subclones was derived from pCPP2067, and pCPP2068 was chosen from this set for further analysis because of the high level of polygalacturonase activity it produced in *E. coli* (Fig. 2). The *pehA* locus in pCPP2068 was further defined by generating a set of

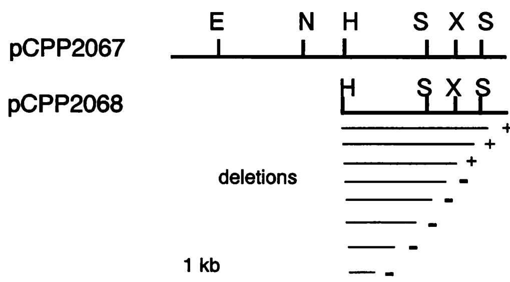

FIG. 2. Restriction map of cloned fragments containing the *pehA* gene. Only restriction sites in the insert DNA are shown. The pCPP2067 DNA insert is in the *Bam*HI site of pBluescript SK(−). The pCPP2068 insert is a *Hin*dIII-*Pst*I subclone from pCPP2067 in pBluescript KS(−). The deletion derivatives were generated by ExoIII digestion of pCPP2068 as described in the text; those marked with a plus produced polygalacturonase activity. Abbreviations: E, *Eco*RI; H, *Hin*dIII; N, *Not*I; S, *Sal*I; X, *Xho*I.

nested deletions through cleavage with *Sac*I and *Bam*HI followed by partial digestion with ExoIII (Fig. 2).

The polygalacturonase produced by *E. coli*(pCPP2067) was compared with that produced by *A. tumefaciens* CG49 on an activity-stained isoelectric focusing gel (Fig. 3). Enzymatic activity could be recovered from sonicated cells but not from extracellular fractions of *E. coli*(pCPP2067) cells, indicating that the polygalacturonase was not secreted by *E. coli*. The enzymes obtained from *E. coli*(pCPP2067) sonic extracts and *A. tumefaciens* CG49 culture fluids displayed the same pI.

Southern blot analysis. Southern blot hybridization was performed to determine whether the *pehA* gene is also present in other *A. tumefaciens* biovars or is unique to biovar 3. Figure 4A shows the autoradiograph of a membrane containing *Hin*dIII-digested DNA from *A. tumefaciens* biovars 1, 2, and 3; *Rhizobium leguminosarum*; and *Bradyrhizobium japonicum*. The blot was probed with the 1.4-kb *Hin*dIII-*Sal*I fragment containing most of the *pehA* locus (Fig. 2), and hybridization was performed at high stringency. A single hybridizing band with a mobility corresponding to 3.5 kb appears in the lane corresponding to biovar 3, whereas no bands appear in any of the other lanes. Identical results were obtained when blots were probed with the 1.4-kb *Hin*dIII-*Sal*I and 1.1-kb *Sal*I-*Sal*I fragments combined (which contain the entire *pehA* locus) and hybridization was performed under low-stringency (50°C) conditions. Therefore, the *pehA* gene seems to be unique to *A. tumefaciens* biovar 3.

Southern blot analysis also provided confirmation that the Tn*5* insertion in CG50 occurred within the 8.5-kb *Eco*RI fragment that contains the structural gene. Figure 4B shows the hybridization pattern of CG49 and CG50 DNA digested with *Eco*RI. A single hybridizing band appears in each lane, but the size changed from 8.5 kb in CG49 to 14.0 kb in CG50 because of the insertion of Tn*5*.

Pathogenicity assays. Root decay was assayed on seedlings of grape cv. Chardonnay. Water-soaked, dark lesions ap-

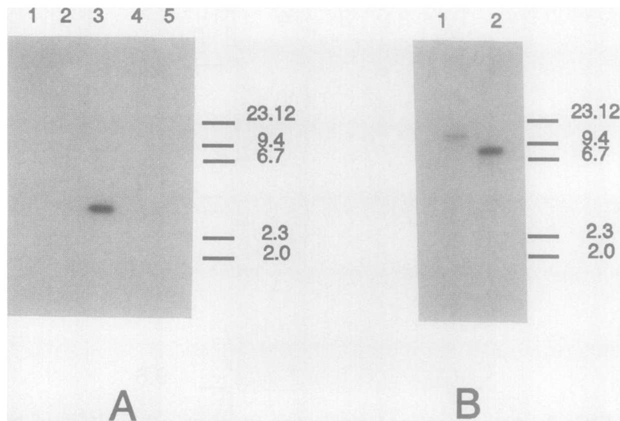

FIG. 4. Southern blot hybridization. DNA was digested with *Hin*dIII (A) or *Eco*RI (B), transferred to an Immobilon membrane (Millipore), probed with the 1.4-kb *Hin*dIII-*Sal*I fragment containing most of the *pehA* gene (Fig. 2), and labeled with [^{32}P]dCTP. High-stringency conditions were used in the wash. (A) *A. tumefaciens* biovar 1 strain C58 (lane 1), *A. tumefaciens* biovar 2 strain R-3 (lane 2), *A. tumefaciens* biovar 3 strain CG49, (lane 3), *R. leguminosarum* (lane 4), and *B. japonicum* (lane 5), (B) *A. tumefaciens* biovar 3 strains CG50 (lane 1) and CG49 (lane 2).

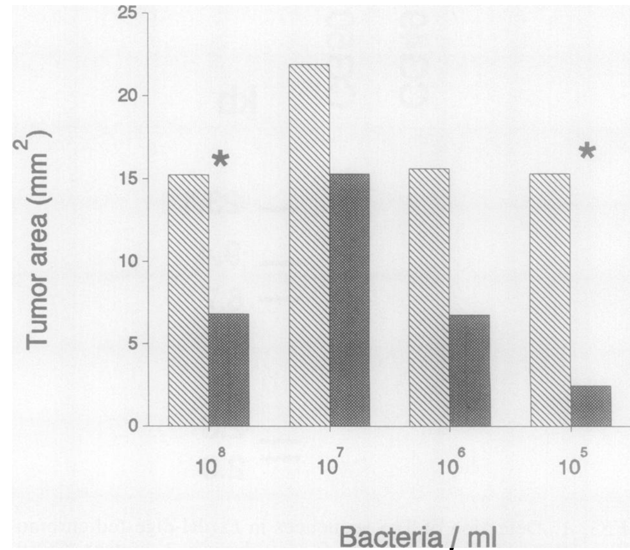

FIG. 6. Tumor induction by CG49 and CG50. Woody stems of grape cuttings cv. Chardonnay were inoculated with CG49 (▧) and CG50 (▩) at four sites in four independent tests with different concentrations of inoculum. Tumors appearing 2 months later were measured. The average tumor surface areas induced by CG49 and CG50 were compared by using a Student *t* test. Differences significant at the 95% confidence level are denoted with an asterisk.

peared within 48 to 72 h after inoculation with CG49; however, no decay was observed following inoculations with CG50 (Fig. 5).

Tumorigenicity assays were performed both on potato discs and on woody stems of grape cv. Chardonnay. There were no differences in the numbers of tumors that developed on potato tuber discs inoculated with different concentrations of CG49 compared with the numbers on those inoculated with CG50. For both strains, an average of 1.4 tumors per potato disc developed at an inoculum concentration of 10^8 CFU/ml.

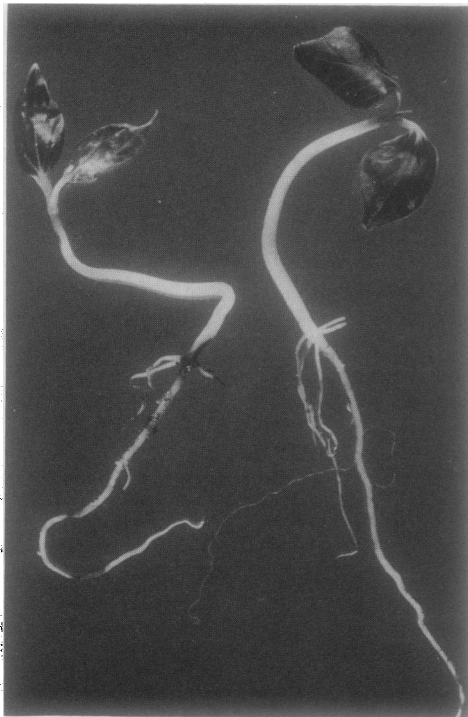

FIG. 5. Root decay in grape seedlings inoculated with *A. tumefaciens* biovar 3. Seedlings of grape cv. Chardonnay were inoculated with strain CG49 (left) and with the polygalacturonase-deficient mutant CG50 (right). The seedlings were photographed 48 h after inoculation.

Tumor induction by CG49 and CG50 in adult grape plants at different inoculum levels was compared. Stems were inoculated at four sites, and four replications were used for each strain and inoculum level. Average tumor areas were compared by using a Student *t* test. As shown in Fig. 6, CG50 induced a smaller tumor area in all cases, and the differences were statistically significant at the 95% level in two of the dilutions. Most notably, the surface area of the tumors was sixfold less for CG50 than for CG49 when the inoculum was at 10^5 bacteria per ml.

DISCUSSION

A. tumefaciens biovar 3 is unique among the *A. tumefaciens* biovars in its host specificity for grapes, in which it causes not only crown gall but also a decay of the roots and systemic infections of the vascular system. Biovar 3 strains are also unique in their ability to produce polygalacturonase. By constructing and analyzing a Peh$^-$ Tn*5* mutant, we have shown that polygalacturonase production is required for root decay and contributes significantly to tumorigenicity in grapes.

The hypothesis that polygalacturonase is a primary determinant of root decay is supported by (i) the established importance of pectic enzymes in the maceration of dicot plant tissues (13), (ii) the previous observation that lesions on roots inoculated with *A. tumefaciens* biovar 3 strain 49 contain polygalacturonase activity with properties similar to those of the polygalacturonase produced by the bacterium in

culture (19), and (iii) the present observation that a polygalacturonase-deficient mutant can no longer produce root decay.

Several aspects of the root decay remain unresolved. For example, *A. tumefaciens* biovar 3 is not known to produce decay in the roots of any plants other than grapes. We do not know whether this is the result of grape-specific regulation or activity of the enzyme or of factors unrelated to polygalacturonase production. Similarly, the role of the enzyme in the invasion of the vascular system has not yet been explored, although we do know that the enzyme is insufficient for bacterial penetration of healthy roots: wild-type strain CG49 does not produce disease in unwounded roots (5).

The observation that the Peh$^-$ mutant CG50 was diminished in its tumorigenicity on grape cuttings was unexpected. It is important to note, however, that the mutant possessed wild-type tumorigenicity on potato and tumorigenicity is not completely abolished on grapes when intermediate levels of inoculum were used. Polygalacturonase is not absolutely required for tumorigenicity in biovar 3, and our work suggests that biovar 1 and 2 strains neither produce extracellular polygalacturonase activity (19) nor harbor genes that are closely related to *pehA*. Nevertheless, polygalacturonase can be conceived to contribute quantitatively to the tumorigenicity of biovar 3 in one or more of the following steps in the infection process: (i) multiplication in wound sites, (ii) bacterial attachment to host cells, (iii) induction of *vir* genes, and (iv) transfer of T-DNA through the host cell wall.

We have observed that the Peh$^-$ mutant CG50 has a diminished ability to multiply in wound sites on grape root seedlings (5), but multiplication in inoculated stem tissues preceding tumor formation remains to be investigated. Similarly, CG50 attached less efficiently to grape roots than did the wild-type strain (5). Attachment ability has been correlated with tumorigenicity, and there is substantial evidence that the saturable receptor for *A. tumefaciens* binding is in the pectic fraction of the plant cell wall (2). Thus, it is possible that cell wall degradation by polygalacturonase creates more binding sites or in some other way enhances binding. Polygalacturonase may also increase virulence by enhancing induction of the *vir* genes. Sugar-mediated induction of the *vir* genes has recently been reported by Nester and coworkers (1, 10). Most importantly, D-galacturonic acid, a potential product of the *A. tumefaciens* biovar 3 polygalacturonase, is the monosaccharide with the strongest effect on *vir* induction at low concentrations (1). Finally, polygalacturonase could have a role in facilitating T-DNA transfer through the plant cell wall, although there are presently no data to support this.

Pectic enzymes have been shown to have a variety of effects on plant tissues and to be important determinants in diseases involving host tissue maceration (13). However, no role in host specificity has ever been demonstrated for a pectic enzyme. Our observations with *A. tumefaciens* biovar 3 raise the possibility that polygalacturonase is a key factor in the specificity of the pathogen for grapes and that the regulation and/or activity of the enzyme is adapted to the host. This is particularly true for the root decay caused by *A. tumefaciens* biovar 3, because its occurrence is independent of the presence of Ti plasmids. Although, the host range of *Agrobacterium* strains as crown gall pathogens has already been shown to be controlled, at least in part, by Ti plasmids (2), our data suggest that polygalacturonase may contribute in a host-specific manner to the tumorigenicity of biovar 3 on grapes. The cloned *pehA* gene should enable us to test these possibilities by monitoring regulation in planta with reporter fusions, by using *pehA* subclones to produce purified polygalacturonase whose effects on host and nonhost tissues can be determined, and by observing the effects on grape pathogenicity of heterologous production of polygalacturonase in other biovars.

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