Regulation of the expression of a *pelA::uidA* fusion in *Erwinia chrysanthemi* and demonstration of the synergistic action of plant extract with polygalacturonate on pectate lyase synthesis

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The phytopathogenicity of *Erwinia chrysanthemi* is chiefly supported by the production of pectate lyase isoenzymes, encoded by the *pel* genes. One of these enzymes, PelA, encoded by the *pelA* gene, seems to represent only a small part of the total pectate lyase activity, but is required for full bacterial pathogenicity. To study the regulation of *pelA* gene expression, a *pelA::uidA* gene fusion was constructed. Expression of this fusion was analysed under various growth conditions and in various genetic backgrounds. Whatever the culture conditions, the *pelA* gene was weakly expressed. Moreover, *pelA* expression seems not to be regulated by the *pecS* gene product, but essentially by the *kdgR* gene product. In many plant-associated bacteria, genes involved in pathogenicity are induced by certain plant compounds. In this work, we demonstrate that *E. chrysanthemi pel* genes are induced in the presence of plant extracts, but only in synergy with known pectate lyase inducers (KDG: 2-keto-3-deoxygluconate; DKII: 2,5-diketo-3-deoxygluconate). However, different *pel* genes did not exhibit the same sensitivity to plant signal molecules. Partial purification of inducing plant compounds suggested that plant extracts contain at least one molecule involved in pectate lyase induction. This compound is thermoresistant, and has a low molecular mass and a very hydrophilic behaviour.

Introduction

Erwinia chrysanthemi is a phytopathogenic bacterium causing soft-rot disease on many plant species. Its pathogenesis is supported by the production and secretion of a battery of depolymerizing enzymes such as pectinases, cellulases and proteases (Collmer & Keen, 1986). Pectin is a major component of plant cell wall material and its degradation leads to maceration of plant tissues. *E. chrysanthemi* strain 3937 produces and secretes one pectin methylesterase and five pectate lyase (Pel) isoenzymes, named PelA to PelE, with isoelectric points ranging from 4·5 to 9·2 (Bertheau *et al.*, 1984). These enzymes are secreted into the external medium, where they cleave pectin to generate unsaturated digalacturonate. This degradation product enters the bacterial cell where it is further degraded through a complex enzymic pathway (Fig. 1). The five different *pel* genes, organized

into two clusters, encode the five pectate lyase isoenzymes (Hugouvieux-Cotte-Pattat *et al.*, 1989; Kotoujansky *et al.*, 1985; Reverchon *et al.*, 1985). One cluster contains the *pelB* and *pelC* genes which encode the neutral isoenzymes (PelB and PelC); the other cluster contains *pelA*, *pelD* and *pelE* genes which encode the acidic isoenzyme (PelA) and the basic isoenzymes (PelD and PelE).

All the genes involved in pectin degradation, including these five pectate lyase genes, constitute independent transcriptional units. Expression of these genes is induced in the presence of polygalacturonate (PGA) or galacturonate. The true intracellular inducers, 2-keto-3-deoxygluconate (KDG) and 2,5-diketo-3-deoxygluconate (DKII), are catabolic products of galacturonate or PGA (Fig. 1) (Chatterjee *et al.*, 1985; Collmer & Bateman, 1981; Condemine *et al.*, 1986; Hugouvieux-Cotte-Pattat & Robert-Baudouy, 1987). Inducibility by these pectic derivatives is mediated by the product of *kdgR*, a negative regulatory gene (Condemine & Robert-Baudouy, 1987; Reverchon *et al.*, 1991). *kdgR* seems to be the only regulatory gene controlling expression of genes coding for the intracellular steps of the pectin

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Abbreviations: DKII, 2,5-diketo-3-deoxygluconate; KDG, 2-keto-3-deoxygluconate; PGA, polygalacturonate.

Fig. 1. Degradative pathway of pectin and galacturonate in *E. chrysanthemi*. The different steps are catalysed by the products of the genes specified near the corresponding arrow: *pem*, gene coding pectin methylesterase; *pel*, pectate lyase (actually five genes: *pelA* to *pelE*); *ogf*, oligogalacturonate lyase; *kduI*, 5-keto-4-deoxyuronate isomerase; *kduD*, 2-keto-3-deoxygluconate oxidoreductase; *uxaC*, uronate isomerase; *uxaB*, altronate oxidoreductase; *uxaA*, altronate hydrolase; *kdgK*, 2-keto-3-deoxygluconate kinase; *kdgA*, 2-keto-3-deoxy-6-phosphogluconate aldolase. The transport systems mediating the entry of galacturonate and 2-keto-3-deoxygluconate into the cell are also indicated (*exuT* and *kdgT*, respectively).

degradative pathway. In contrast, synthesis of pectin methylesterase and pectate lyase is under the control of many other regulatory genes (Condemine & Robert-Baudouy, 1987; Hugouvieux-Cotte-Pattat *et al.*, 1986; Hugouvieux-Cotte-Pattat & Robert-Baudouy, 1989; Reverchon *et al.*, 1990). Recently, one of them, *pecS*, has been characterized, and seems to negatively control *pel* gene expression only (Reverchon *et al.*, 1990).

Pathogenicity tests performed on *Saintpaulia* plants *in vitro* suggest that the genes of the *pelBC* cluster are not essential for bacterial pathogenicity (Boccaro *et al.*, 1988). In contrast, each of the enzymes produced by the *pelADE* gene cluster is required for pathogenicity (Boccaro *et al.*, 1988). The two alkaline pectate lyases PelE and PelD, which correspond to approximately 60% and 15% of total pectate lyase activity, respectively, exhibited strong plant tissue macerating activity. The unique acidic pectate lyase PelA of *E. chrysanthemi* represents 3% of total pectate lyase activity and shows a slight maceration ability (Barras *et al.*, 1987; Garibaldi & Bateman, 1971; Tamaki *et al.*, 1988). However, pathogenicity of *E. chrysanthemi pelA* mutants is strongly

decreased. Regarding the weak contribution of PelA to total Pel activity and its crucial importance in *E. chrysanthemi* pathogenicity, we wondered whether weak PelA activity is related to weak transcription of the *pelA* gene and if the PelA protein could have another major enzymic activity. This led us to construct a *pel::uidA* fusion and to follow its expression in various growth conditions and in many genetic backgrounds.

The interaction between plant and pathogen is a dynamic process involving signal exchange between interacting organisms. Indeed, for most plant-associated bacteria it has been shown that many bacterial genes involved in plant-bacteria interaction are induced by plant molecules. For example, the expression of the *Agrobacterium vir* genes that govern virulence is induced by acetosyringone and other plant phenolic compounds (Bolton *et al.*, 1986; Stachel *et al.*, 1985, 1986). The *nod* genes of *Rhizobium* species, which control nodulation mechanisms, are induced by some plant compounds identified as flavonoids (Downie & Johnston, 1988; Redmond *et al.*, 1986). Consequently, we investigated the effect of plant compounds such as acetosyringone,

Table 1. *Bacterial strains, plasmids and phages*

Strain	Genotype/description*	Source/reference
<i>Erwinia chrysanthemi</i>		
A350	<i>lmrT^c lacZ</i> ; used as wild-type strain	Hugouvieux-Cotte-Pattat & Robert-Baudouy (1985)
A1696	<i>lmrT^c lacZ pelA::uidA(kan)</i>	This work
A1702	<i>lmrT^c lacZ pelA::uidA(kan) arg ogl</i>	
A1688	<i>lmrT^c lacZ pelA::uidA(kan) arg kdgK</i>	
A1698	<i>lmrT^c lacZ pelA::uidA(kan) kduD::Mu(Ap, lac)</i>	
A1697	<i>lmrT^c lacZ pelA::uidA(kan) kdgR</i>	
A1699	<i>lmrT^c lacZ pelA::uidA(kan) pecS::Mu(Cm)</i>	
A1808	<i>lmrT^c lacZ pelB::uidA(kan)</i>	
A1812	<i>lmrT^c lacZ pelC::uidA(kan)</i>	
A1813	<i>lmrT^c lacZ pelD::uidA(kan)</i>	N. Hugouvieux-Cotte-Pattat and others (unpublished)
A1828	<i>lmrT^c lacZ pelE::uidA(kan)</i>	
<i>Escherichia coli</i>		
NM522	$\Delta(lac-proAB)$ <i>thi hsd5 supE F'(proAB⁺, lacI^q, lacZΔM15)</i> Stratagene	
Phages		
PhiEC2	General transducing phage of <i>E. chrysanthemi</i>	Resibois <i>et al.</i> (1984)
Plasmids		
pBluescript	Ap, 'lacZ	Stratagene
pBR325	Ap, Tc, Cm	Bolivar (1978)
p19014	Plasmid harbouring insertional <i>uidA(kan)</i> cassette	N. Bardonnet (unpublished)
pBLI625	pBluescript derivative harbouring <i>pelE</i> and <i>pelA</i> genes	This work
pBRSE5	pBR325 derivative harbouring downstream region and first codons of <i>pelA</i> gene	This work
pAUID5	pBR325 derivative harbouring <i>pelA::uidA(kan)</i> fusion	This work

* Genotype symbols are according to Bachmann (1990). *lmrT^c* indicates that the transport system encoded by the *lmrT* gene, which mediates entry of lactose, melibiose and raffinose into the cells, is constitutively expressed.

and of crude plant extracts, on the expression of *pel* genes and especially on the *pelA* gene.

Methods

Bacterial strains, plasmids and culture conditions. Bacterial strains and plasmids used in this study are shown in Table 1. Cells were grown in complete rich LB medium or in synthetic M63 medium (Miller, 1972). When required, media were supplemented with glycerol (2 g l⁻¹) as carbon source, amino acids (40 mg l⁻¹), and with antibiotics at the following concentrations: kanamycin, 20 µg ml⁻¹; ampicillin, 50 µg ml⁻¹; tetracycline, 10 µg ml⁻¹; chloramphenicol, 20 µg ml⁻¹. Media were solidified with Difco agar (15 g l⁻¹). Polygalacturonate (0.25%, w/v) was added to culture media as a pectate lyase inducer.

Enzyme assays. Pectate lyase and β -glucuronidase assays were performed on toluenized extracts of cells grown to the late exponential growth phase. To avoid catabolic repression of pectate lyase synthesis, cultures were grown in M63 minimal medium supplemented with glycerol as carbon source. Pectate lyase activity was determined by measuring the degradation of polygalacturonate into unsaturated products that absorb at 235 nm (Moran *et al.*, 1968); specific activity was expressed in µmol unsaturated products liberated min⁻¹ (mg bacterial dry weight)⁻¹. β -Glucuronidase activity was assayed by using *p*-nitrophenyl β -D-glucuronide as substrate (Novel *et al.*, 1974). The degradation product, *p*-nitrophenol, was detected at 405 nm (ϵ = 0.0045). β -Glucuronidase specific activity was expressed in nmol *p*-nitrophenol liberated min⁻¹ (mg bacterial dry weight)⁻¹.

Genetic techniques and DNA methods. Transduction with phage phiEC2 was performed according to Resibois *et al.* (1984). Marker-exchange recombinations were obtained after successive cycles of

growth in a low-phosphate medium (Roeder & Collmer, 1985), supplemented for the first culture with 1% (v/v) plant extract. Standard molecular cloning techniques used in this study (small- and large-scale plasmid DNA extraction, restriction enzyme digestion, agarose gel electrophoresis, DNA ligation, transformation and electroporation of bacterial cells) were performed as described by Ausubel *et al.* (1987) and Sambrook *et al.* (1989).

The *uidA-kan* cassette was provided by N. Bardonnet (unpublished results). The *uidA* gene was isolated from *Escherichia coli* and codes for β -glucuronidase activity, which is easy to detect and to assay. This gene was deleted of its own promoter and cloned in an insertional cassette including kanamycin resistance (*kan*) as marker gene.

Plant extract preparation and partial purification of inducing factors. Crude plant extracts were obtained by crushing plant organs (leaves and stems of *Diffenbachia*, fruit of pumpkin, or carrot roots). The resulting juices were clarified by centrifugation and subsequent filtrations on membranes of decreasing porosity. Extracts were sterilized by final filtration on a 0.22 µm porosity membrane and stored in aliquots for several months at -20 °C.

For partial purification, plant extracts were first filtered on a Centrikon 10 device (Amicon) which eliminates molecules of molecular mass higher than 10 kDa. The filtered solution was fractionated on a Fractogel column with a 5 kDa threshold (TSK 40, Merck). Elution was done with 20 mM-NH₄OH and all the fractions were tested for their inducing properties. Inducing fractions of different runs were pooled, concentrated in a vacuum dryer and subsequently fractionated on HPLC columns. The first column used was an NH₂ type (Spherisorb S5 NH₂) and elution was performed with an acetonitrile/H₂O gradient. As below, each fraction was tested and inducing ones were pooled and concentrated. The pool of inducing fractions was finally loaded on a hydrophobic interaction column (C18 type: Lichosorb L10 C18 25R) and eluted with an acetonitrile/acetic acid/H₂O gradient. Each fraction

was tested for its inducing property. Inducing fractions of different runs were pooled and concentrated for characterization.

Results

Construction of the *E. chrysanthemi* mutant containing the *pelA::uidA* fusion

The transcriptional *pelA::uidA* fusion was constructed by insertion of a *uidA-kan* cassette into the open reading frame of the *pelA* gene (Fig. 2). Since the *uidA* promoter was deleted, expression of this reporter gene is dependent on *pelA* regulatory elements.

The 4.5 kb *HindIII*–*PvuII* fragment, harbouring the *E. chrysanthemi pelA* and *pelE* genes, was cloned in the *HindIII*–*SmaI* sites of pBluescript KS+, to produce pBLI625. Then the 2 kb *EcoRI* fragment harbouring the regulatory region and the beginning of the coding region of *pelA* was subcloned from pBLI625 into the *EcoRI* site of pBR325, giving pBRSE5. A *uidA-kan* cassette, obtained by *SmaI* digestion of p19014, was introduced into the unique *HpaI* site of pBRSE5. The resulting plasmid pAUID5, containing the *pelA::uidA* fusion, was introduced into *E. chrysanthemi* strain A350 by electroporation. Recombination of the *pelA::uidA* fusion in the chromosome was performed by successive cultures in low-phosphate medium. We obtained 10 recombinants with the correct antibiotic resistance pattern: kanamycin resistance (*kan* is the fusion marker), ampicillin and tetracycline sensitivity (segregation of the pBR325 derivative replicon). The Pel pattern of these recombinants was then analysed by isoelectric focusing. Seven of the 10 recombinants had lost PelA isoenzyme activity and corresponded to *pelA* mutants (Fig. 3). A Southern hybridization experiment confirmed that the *pelA::uidA* fusion was correctly recombined in these seven mutants (data not shown).

Fig. 2. Construction of *pelA::uidA* fusions. The successive plasmids for the construction of the fusion are represented in a linear form. The derived vector is indicated on the right-hand side of the plasmid; arrows represent gene extent and transcription direction.

Expression of the *pelA::uidA* fusion in various growth conditions

The *pelA::uidA* mutant A1696 was analysed for β -glucuronidase synthesis in the absence and presence of PGA as inducer and also in the presence of plant extract to test possible induction by plant molecules (Table 2). Pectate lyase residual activity corresponding to the global activities of PelB, PelC, PelD and PelE was also determined in the same growth conditions (Table 2). The *pelA::uidA* fusion was poorly expressed in all conditions tested. A very weak induction was observed with PGA. A better induction could be detected in the presence of both PGA and plant extract (Table 2). Therefore it seems that the weak contribution of PelA to the total Pel activity is really related to weak transcription of *pelA* and is probably not due to another major enzymic activity of the PelA protein. Moreover, Pel residual activity is not affected by the lack of the *pelA* gene product, confirming that PelA activity contributes a very small part of the total Pel activity in all conditions tested.

pelA::uidA expression in different genetic backgrounds

By using the transducing phage phiEC2, the *pelA::uidA* fusion was transduced into different mutants of the pectinolytic pathway: *ogl*, *kduD* and *kdgK*. Transductants were checked for their antibiotic resistance pattern and the loss of PelA activity on an electrofocusing gel. β -Glucuronidase was assayed on cultures grown without inducers, with PGA and with PGA supplemented with plant extract (Table 3).

In the presence of PGA, an *ogl* mutant accumulates digalacturonates and is unable to produce true Pel inducers (KDG and DKII) (Chatterjee *et al.*, 1985; Reverchon & Robert-Baudouy, 1987a). In the *ogl* mutant, the *pelA* fusion was not induced by PGA or PGA plus plant extract (Table 3), indicating that *pelA* induction also requires the degradation of digalacturonate.

In *kduD* or *kdgK* mutants, addition of PGA to the culture medium resulted in a significant induction of *pelA* expression (Table 3), suggesting that *pelA* induction is also sensitive to the DKII and KDG accumulation that occurs in these mutants (Chatterjee *et al.*, 1985; Condemine *et al.*, 1986; Hugouvieux-Cotte-Pattat & Robert-Baudouy, 1987).

The *pel::uidA* fusion was also transduced into *kdgR* and *pecS* regulatory mutants. The *kdgR* gene product regulates all the pectinolytic pathway genes (Hugouvieux-Cotte-Pattat & Robert-Baudouy, 1989; Reverchon & Robert-Baudouy, 1987b), and *pecS* is another negative regulatory gene with a narrow range, since it controls only *pel* gene expression and has no action on the intracellular steps of pectinolysis

Fig. 3. Isoelectric focusing of pectate lyase enzymes present in culture supernatant of different strains. 1, Supernatant of strain A350 (wild-type) cultivated in the presence of PGA. 2 and 3, supernatant of strain A1696 (*pelA::uidA*) cultivated in the absence and presence of PGA, respectively.

Table 2. Expression of a *pelA::uidA* fusion in various growth conditions

Bacteria were grown in M63 minimal medium with glycerol (2 g l⁻¹) as carbon source. PGA and plant extract were added at 0.25% (w/v) and 1% (v/v), respectively. Pectate lyase activity is expressed in μmol unsaturated product liberated min⁻¹ (mg bacterial dry weight)⁻¹. β -Glucuronidase activity is expressed in nmol *p*-nitrophenol liberated min⁻¹ (mg bacterial dry weight)⁻¹. ND, Not detectable.

Strain	Inducer	Pectate lyase activity	β -Glucuronidase activity
A1696 (<i>pel::uidA</i>)	None	0.01	2
	PGA	0.07	5
	PGA + carrot extract	2.22	7
A350 (wild-type)	None	0.01	ND
	Carrot extract	0.02	ND
	PGA	0.09	ND
	PGA + carrot extract	2.29	ND
	Galacturonate (GalA)	0.16	ND
	GalA + carrot extract	1.18	ND

(Reverchon *et al.*, 1990). In a *kdgR* mutant, *pelA* showed the same constitutive expression whatever the culture conditions (Table 3). The level of *pelA* expression was nearly equivalent to the maximum induced level observed

Table 3. Expression of a *pelA::uidA* fusion in various genetic backgrounds

Bacteria were grown in M63 minimal medium with glycerol (2 g l⁻¹) as carbon source. PGA and plant extract were added at 0.25% (w/v) and 1% (v/v), respectively. See Table 2 for units of enzyme activity.

Strain	Inducer	Pectate lyase activity	β -Glucuronidase activity
A1696 (<i>pelA::uidA</i>)	None	0.01	2
	PGA	0.07	5
	PGA + carrot extract	2.22	7
A1702 (<i>pelA::uidA, ogI</i>)	None	0.02	3
	PGA	0.04	3
	PGA + carrot extract	0.14	3
A1698 (<i>pelA::uidA, kduD</i>)	None	0.02	3
	PGA	13.50	22
	PGA + carrot extract	8.31	14
A1688 (<i>pelA::uidA, kdgK</i>)	None	0.02	3
	PGA	16.90	20
	PGA + carrot extract	21.90	13
A1697 (<i>pelA::uidA, kdgR</i>)	None	0.46	27
	PGA	0.94	27
	PGA + carrot extract	2.72	19
A1699 (<i>pelA::uidA, pecS</i>)	None	0.24	5
	PGA	1.20	6
	PGA + carrot extract	6.33	4

Table 4. Influence of plant extract concentration on *Pel* synthesis

Bacteria were grown in M63 minimal medium with glycerol (2 g l⁻¹) as carbon source. PGA was added at 0.25% (w/v), and carrot extract was added as specified. See Table 2 for units of pectate lyase activity.

Plant extract (% v/v)	Pectate lyase activity	Induction ratio
None	0.09	
0.2	0.13	1.4
1.0	2.03	22.5
2.0	1.83	20.3
4.0	1.80	20.0
8.0	1.90	21.1
12.0	1.28	14.2

in *kdgK* or *kduD* mutants. These results suggest that the *kdgR* gene product is the major regulatory protein responsible for *pelA* repression in absence of inducer. The *pelA::uidA* fusion had approximately the same expression in a *pecS* mutant as in the wild-type strain (Table 3). This suggests that *pecS* has no significant effect on *pelA* expression. In contrast with the other *pel* genes,

Table 5. Effect of plant extract on the expression of the different *pel::uidA* fusions

Bacteria were grown in M63 minimal medium with glycerol (2 g l⁻¹) as carbon source. PGA and plant extract were added at 0.25% (w/v) and 1% (v/v), respectively. See Table 2 for units of enzyme activity.

Strain	Inducer	Pectate lyase activity	β -Glucuronidase activity	Induction ratio
A1696 (<i>pelA::uidA</i>)	None	0.01	2	
	PGA	0.07	5	
	PGA + carrot extract	2.22	7	3.5
A1808 (<i>pelB::uidA</i>)	None	0.01	22	
	PGA	0.04	37	
	PGA + carrot extract	2.49	167	7.5
A1812 (<i>pelC::uidA</i>)	None	0.10	15	
	PGA	0.14	25	
	PGA + carrot extract	2.71	284	18.9
A1813 (<i>pelD::uidA</i>)	None	0.02	25	
	PGA	0.03	37	
	PGA + carrot extract	0.89	1560	62.5
A1828 (<i>pelE::uidA</i>)	None	0.01	708	
	PGA	0.05	672	
	PGA + carrot extract	0.46	1260	1.8

expression of *pelA* might not be regulated by *pecS*, confirming that *kdgR* is the essential regulatory gene for *pelA*.

Effect of plant extract on total Pel synthesis

In the wild-type strain, PGA induced Pel synthesis only ninefold when added to culture medium (Table 2). Plant extract alone had no significant effect on Pel synthesis. However, the presence of both plant extract and PGA had a strong inducing effect on Pel synthesis: the induction ratio was about 230-fold in the presence of 1% plant extract (Table 2). The same superinduction phenomenon was observed in the presence of galacturonate and plant extract (Table 2). Thus plant extract had a significant effect on Pel induction only in synergy with known pectic derivative inducers (KDG, DKII).

The effect of plant extract concentration on Pel synthesis was analysed in the presence of PGA. Induction ratios increased with the plant extract concentration to a maximum with 1% of plant extract (Table 4). With higher concentrations, the induction ratio decreased. This could be due to catabolite repression exerted by sugars present in the plant extract, or to the presence of Pel inhibitors in the extract. Extracts from *Diffenbachia* stems and leaves, pumpkin fruit and carrot roots gave similar inducing effects (data not shown), suggesting that

the potential coinducing factor(s) is (are) widely distributed among plant species and tissues.

Effect of the plant extract in various genetic backgrounds

As shown above, the *pelA* gene product has only a very small effect on Pel global activity; for this reason Pel residual activity in the *pelA* mutant was equivalent to the Pel global activity in all the genetic backgrounds tested.

In an *ogl* mutant, PGA had no significant effect on Pel synthesis, and PGA with plant extracts showed only a very small inducing effect (Table 3). This induction was probably due to the presence in plant extract of small amounts of inducing compounds such as galacturonate.

When PGA was added to the culture medium of *kduD* or *kdgK* mutants, accumulation of larger amounts of true inducers led to superinduction of Pel synthesis (Table 3). In these mutants, addition of plant extract had no significant effect on Pel synthesis, indicating that accumulation of true inducers in *kduD* or *kdgK* mutants might lead to the full expression of *pel* genes which cannot be increased by plant extract.

In *kdgR* and *pecS* mutants, high constitutive Pel expression was observed. However, Pel synthesis was still inducible by plant extract (Table 3).

Effect of plant extract on the expression of the different *pel::uidA* fusions

We compared the expression of the five *pel* genes by using *uidA* fusions with each *pel* gene. As for the *pelA::uidA* fusion, the same *uidA-kan* cassette was inserted into the open reading frame of each *pel* gene. The resulting constructions were introduced into the chromosome of *E. chrysanthemi* by homologous recombination. The β -glucuronidase activity corresponding to the expression of the *pelA::uidA*, *pelB::uidA*, *pelC::uidA*, *pelD::uidA* and *pelE::uidA* fusions was determined under various culture conditions, including the presence of plant extract (Table 5). Pel residual activity was also determined in these mutants to assess the contribution of the missing Pel isoenzyme (Table 5).

Expression of the *pelD::uidA* and *pelE::uidA* fusions in plant-extract-induced conditions was very high in comparison with other fusions. Consequently, Pel residual activity was significantly reduced in these mutants. Expression of the *pelD::uidA* fusion was strongly induced (62-fold) in the presence of plant extract. In contrast, the basal expression of the *pelE::uidA* fusion was already high in non-induced conditions, and plant extract induced expression only 1.8-fold. Plant extract was also a poor inducer of *pelA::uidA* expression and had a fairly low effect on *pelB::uidA* and *pelC::uidA* expression.

Fig. 4. Partial purification of plant inducing molecules. The elution profile (—) and the inducing activity of the fractions (■) are shown. The elution profile was obtained at 260 nm; the total scale represents 0.2 optical density units. Fractions with a significant inducing activity were found in two peaks, indicated on the figure.

Partial purification of plant extract inducing factors

Plant phenolic compounds or flavonoids are known to induce expression of genes involved in plant–bacteria interaction, such as the *vir* genes in *Agrobacterium* (Bolton *et al.*, 1986; Stachel *et al.*, 1985, 1986) and the *nod* genes in *Rhizobium* (Downie & Johnston, 1988; Redmond *et al.*, 1986). To test if the inducing factor in plant extract was one of these compounds, some of these molecules (acetosyringone, coumarin, hydroxycoumarin, catecholamine and apigenin) were analysed in a wide range of concentrations (from 10^{-7} M to 0.5 M) for their ability to induce or to enhance *pel* expression, in the presence or absence of PGA (data not shown). None of them showed an inducing or enhancing effect at any concentration, suggesting that the nature and structure of molecules inducing *pel* gene expression in *E. chrysanthemi* might be different from those of *Agrobacterium* or *Rhizobium*.

Combustion (1 h at 800 °C) destroyed the inducing activity, indicating that the factor(s) is organic. The *pel*-inducing factor of plant extract is thermoresistant since it could be autoclaved without loss of activity. As estimated by membrane filtration, the inducing factor is smaller than 10 kDa. During the first step of purification, which was a fractionation on an exclusion column, the inducing fractions were grouped in a unique wide peak, with an apparent molecular mass between 350 and 1000 Da. Therefore, the plant signal molecules have a relatively small molecular mass, and cannot be molecules such as PGA or pectins, nor proteins or large polymers.

A further step of purification was performed by HPLC on an NH_2 column. Two distinct peaks of inducing compounds were obtained (Fig. 4). The major part of activity was in the first peak. This result suggested the presence in plant extract of either several inducing molecules, or only one compound which could exist in several distinct chemical or structural conformations. The two inducing peaks did not absorb significantly at

260 nm, confirming that the inducing factor(s) were not phenolic or flavonoid compounds.

Fractions of the first peak, which gave the stronger inducing effect, were pooled and subsequently fractionated on a hydrophobic interaction column (C18). Only the first fraction of the elution had inducing activity. This result demonstrated that the active molecule has a very hydrophilic behaviour. During concentration of the active fraction by vacuum, the inducing factor precipitated and we failed to redissolve it. However, the precipitate still had inducing activity, but it was impossible to characterize this compound by an analytical method such as NMR. Work is in progress in our laboratory to solubilize the active factor, to allow further purification and characterization of this molecule.

Discussion

The weak expression of the *pelA::uidA* fusion in all conditions tested indicates a low level of transcription. This result can be related to the presence of bad transcription signals in the *pelA* regulatory region (Favey *et al.*, 1992). Analysis of *pelA* expression in mutants blocked in different steps of the pectin degradative pathway demonstrates that *pelA* induction, like the induction of other *pel* genes, requires the formation of DKII or KDG. Moreover, we demonstrated that *pelA* expression is regulated by the *kdgR* gene product, as suggested by the presence of a typical KdgR-box in the *pelA* regulatory region (Favey *et al.*, 1992). However, in contrast to the global Pel synthesis, the regulatory gene *pecS* did not seem to control *pelA* expression. Regarding weak *pelA* expression, it was impossible to explain the requirement of *pelA* for pathogenicity in terms of pectate lyase activity. These results allow Pel activity and pathogenicity to be distinguished. However, the requirement of *pelA* in pathogenicity could be related to a particular expression of that gene during the infection process. A gene, *pecX*, whose product seems to modulate

pelA expression, has been detected in the region upstream of the *pelA* gene (Reverchon *et al.*, 1986). Preliminary sequence data indicate that the *pecX* product has some homology with a globin, and therefore could be involved in oxygen metabolism (S. Favay and others, unpublished results). These results suggest that PelA synthesis could be controlled by other factors, still unknown, such as oxygen concentration. The *Agrobacterium virG* gene has two promoters; one of them is inducible by a variety of environmental stresses (Mantis & Winans, 1992; Winans, 1990). In the same way, *pelA* expression could be induced by various types of stress, such as oxygen limitation, which could act as important signals for the expression of *E. chrysanthemi* pathogenicity. The development of a disease caused by *Erwinia* strongly depends on the contamination levels but also on environmental conditions such as temperature, humidity and oxygen limitation (Perombelon, 1990). Therefore, it would be interesting to follow *pelA* expression during plant infection in different environmental conditions. To allow this investigation, we had to construct a merodiploid strain, to have an intact *pelA* gene required for infection, and a *pelA::uidA* fusion as an activity marker.

We demonstrated that in *E. chrysanthemi*, Pel synthesis is strongly induced in the presence of both PGA and plant extract. The plant inducing effect is visible only in synergy with PGA or galacturonate, indicating that the plant inducing factor is not the natural true inducer like KDG or DKII. However, the presence of KDG or DKII seems to be required for effective action of plant extract of Pel synthesis. In an *ogl* mutant, which lacks the capacity to produce the natural true inducers from PGA, plant extract does not exhibit any inducing activity. In addition, in *kdgR* and *pecS* regulatory mutants, Pel synthesis is still induced in the presence of plant extract, suggesting that induction by plant extract occurs through a mechanism other than *kdgR* or *pecS* derepression. To improve our knowledge of the relationship between plant-mediated induction and other forms of regulation, we are working on the construction of a double *kdgR-pecS* mutant with which to investigate the effect of plant extract.

Plant extract does not have the same effect on the expression of the different *pel* genes. The expression of *pelE* and *pelA* seems to be poorly sensitive to plant extract induction. As previously discussed, *pelA* is probably sensitive to other environmental factors. A strong basal expression of *pelE* was observed, suggesting that the PelE isoenzyme is probably involved in the first steps of infection to allow pectin degradation and formation of inducers in order to initiate the expression of other *pel* genes. Expression of *pelB* and *pelC* is only moderately induced by plant extract, whereas *pelD* gene expression is very sensitive to plant inducing factor.

These observations could be correlated with the absence of evident roles of the PelB and PelC isoenzymes in pathogenicity and with the major contribution of the PelD isoenzyme during plant infection. We can suggest an ecological meaning for that induction mechanism. Pel synthesis is induced by PGA or galacturonate to a sufficient level for saprophytic growth. But, when inducing plant molecules are present, Pel synthesis is strongly induced to a higher level appropriate to rotting plant tissues quickly.

Preliminary characterization experiments indicate that the plant extract contains at least one compound responsible for the inducing effect on Pel synthesis. Total purification and subsequent characterization of active molecules have not been successful because of precipitation of the inducing compound. However, preliminary characterization revealed that the inducing factor is thermoresistant, has a small molecular mass and is neither a phenolic nor a flavonoid compound.

Agrobacterium tumefaciens and *E. chrysanthemi* are both well-studied Gram-negative phytopathogenic bacteria which attack wounded plants. We can establish a parallel between induction mechanisms for virulence genes in each bacterium. Specific monosaccharides and particular phenolic compounds play a synergistic role in *vir* gene induction in *A. tumefaciens* (Cangelosi *et al.*, 1990; Shimoda *et al.*, 1990; Winans, 1991). Likewise, we demonstrated that in *E. chrysanthemi* monosaccharides, KDG and DKII, with unidentified plant molecules also play a synergistic role in pectate lyase induction. In *Agrobacterium*, the VirA protein serves as an environmental sensor to detect the plant signal molecules and transduces this signal to the VirG protein that activates *vir* gene expression (Winans, 1991). It would be interesting to determine if a protein equivalent to the *virA* product exists in *Erwinia* to serve as a plant signal molecule receptor.

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