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Iron Is a Triggering Factor for Differential Expression of *Erwinia chrysanthemi* Strain 3937 Pectate Lyases in Pathogenesis of African Violets

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This work was aimed at investigating possible links between pectinolysis and chrysobactin-dependent iron assimilation, two major determinants of the pathogenicity of *Erwinia chrysanthemi* 3937 on African violets. Transcriptional expression of the relevant pectinase-encoding genes (*pelA* to *pelE* and *pem*) was analyzed during the early stages of pathogenesis by means of GUS fusions. The *pelD*::*uidA* fusion was induced 6 h after inoculation, earlier and in higher levels than *pelB*::*uidA*, *pelC*::*uidA*, *pelE*::*uidA*, and *pem*::*uidA*; *pelA*::*uidA* was not induced. *PelD*::*uidA* expression was compared with that of *pelE*::*uidA* in different mutants. In a *cbr* mutant, derepressed for chrysobactin, *pelD*::*uidA* was weakly expressed, *pelE*::*uidA* expression did not change significantly, and production of the cognate PelD and PelE isoenzymes was reduced. A chrysobactin-deficient *pelD*::*uidA* mutant grew poorly; no *pelD*::*uidA* expression was recovered. In a *kdgR* mutant derepressed for pectinolytic functions, *pelD*::*uidA* expression was the same until 15 h postinoculation, after which a substantial increase was apparent. The expression of *pelD*::*uidA* was stimulated by the presence of iron chelators in the growth medium and the absence of functional chrysobactin-mediated iron uptake. The data provide evidence supporting the central role of iron in plant pathogenesis.

Additional keywords: regulation, siderophore, virulence.

The species *Erwinia chrysanthemi* generates various diseases, such as soft rot, wilting, and dwarfing, on a wide range of plants. Soft rot occurs in hot and humid climates on vegetables and ornamentals of economic importance. It consists of a disorganization of parenchymatous tissues following the release in plant walls of bacterial pectinolytic enzymes (Garibaldi and Bateman 1971; Temsah et al. 1991). Pectinases are secreted via a specific pathway encoded by the *out* operon, whose activity is also essential for the secretion of cellulases (for a review, see Salmond 1994). Out$^-$ mutants, deficient in the secretory function, are nonpathogenic (Andro et al. 1984). No significant role in pathogenesis has been assigned to the cellulolytic enzymes characterized in *E. chrysanthemi* (Boccara et al. 1994).

Strain 3937, isolated from African violets (*Saintpaulia ionantha* H. Wendl.), produces one pectin methyl esterase (PME) encoded by the *pem* gene and five major pectate lyases (Pels) expressed from independent cistrons *pelA* to *pelE*. These cistrons are organized in two clusters on the chromosome, *pelBC* and *pelAED* (Reverchon et al. 1986; Hugouvieux-Cotte-Pattat and Robert-Baudouy 1992). It is noteworthy that Pels, apparently redundant in their enzymatic activity, differ from each other in their pI (Bertheau et al. 1984), their ability to generate diverse pectic oligomers (Preston et al. 1992), and their contribution to pathogenesis (Boccara et al. 1988; Boccara and Chatain 1989).

The aptitude of this pathogen to modulate the expression of its enzymatic equipment, possibly in relation to its immediate environment, indicates a fine tuning mediated at the gene level. In addition, the possibility that Pels exert a dual role because of their ability to both disrupt wall structures and generate elicitors for plant defense (Hahn et al. 1981; Messiaen and Van Cutsem 1993; Messiaen and Van Cutsem 1994) is likely to be important for their regulation in planta. Ex planta studies have shown that the major effector controlling *pel* gene expression is the KdgR protein, which acts as a transcriptional repressor of a set of genes involved in pectinolysis (Reverchon et al. 1991; Nasser et al. 1992; Nasser et al. 1994). In the absence of pectic inducers, KdgR binds to its operator (KdgR box), characterized as a 17-bp palindromic sequence. However, in *kdgR* mutants pectate lyases are still inducible in the presence of pectin derivatives, suggesting the existence of other regulatory factors (Reverchon and Robert-Baudouy 1987). Furthermore, the transcriptional activities of *pel* genes respond differentially to catabolic repression, growth phase, temperature, and nitrogen starvation (Hugouvieux-Cotte-Pattat et al. 1986; Hugouvieux-Cotte-Pattat et al. 1992). Anaerobiosis stimulates the activity of *pelA*, *pelD*, and *pelE*; high osmolarity increases *pelE* expression (Hugouvieux-Cotte-Pattat et al. 1992). Iron limitation induces *pelE*, *pelB*, and *pelC* transcription (Sauvage and Expert 1994). Con-

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sidering the diversity of the environmental factors involved, it would be interesting to know whether all of them have physiological significance in pathogenesis. Of particular interest is the question of which factor might influence the primary phase of soft rot, before production of the first catabolic inducers.

The hypothesis that iron may play a role in planta in modulating the expression of Pels deserves attention. The level of iron in intercellular fluids (IFs) proved to be low enough to slow down the growth of bacterial mutants lacking a functional iron assimilation pathway mediated by chrysobactin (Neema et al. 1993; Masclaux and Expert 1995). Such mutants are affected in the spread of symptoms (Enard et al. 1988). The *fct* gene encoding the ferrichrysobactin outer membrane transport function and the *cbs* genes involved in the four primary steps of chrysobactin biosynthesis define an operon, *fct cbsCEBA* (Franza and Expert 1991), that is strongly repressed by iron (Expert et al. 1992; Masclaux and Expert 1995). In addition, strain 3937 expresses a second iron acquisition system, dependent on the siderophore achromobactin (Mahé et al. 1995), that is induced under conditions of iron deficiency weaker than those required for chrysobactin derepression. Interestingly, mutations affecting ferriachromobactin permease, an ABC transporter encoded by the *cbrABCD* operon, give rise to accumulation of achromobactin in the external medium, thereby decreasing iron availability to the bacterial cells. The result is the derepression of chrysobactin production under conditions normally repressive (Mahé et al. 1995). Originally expected to be impaired in a regulator, *cbr* mutants were defined as chrysobactin regulatory mutants. *Cbr* mutants display delayed symptoms on African violets, whereas their behavior is not changed in later stages of soft rot (Sauvage and Expert 1994).

In this work, we analyze the expression of *pel* and *pem* genes during the early phase of soft rot on potted plants, using *uidA* as a reporter gene. Interestingly, *pelD* appeared to be the first gene induced. Its expression was thus compared with that of *pelE* as a representative iron-regulated *pel* gene, when placed in different mutant backgrounds. A detailed analysis of the different responses is reported. The data are discussed in relation to a previous work showing the regulated response of the chrysobactin *fct cbsCEBA* operon under the same conditions of infection (Masclaux and Expert 1995).

RESULTS

Activity of *pel*:: and *pem*::*uidA* gene fusions in strain L37 after leaf inoculation.

To study the transcriptional activity of *pel* and *pem* genes during pathogenesis, a culture of L37 cells harboring one of the *pelA*::, *pelB*::, *pelC*::, *pelD*::, *pelE*::, and *pem*:: *uidA* fusions was inoculated into the leaf parenchyma of potted plants, as described in Materials and Methods. GUS activity, produced by the bacteria present in extractable fluids, was assayed at 3-h intervals over 24 h and then every 12 h after inoculation. The growth of *pel* mutants was compared with that of the wild-type strain. Figure 1A shows that the growth rate of mutant and parental strains was similar during the first 48 h following inoculation. After this period of time, the growth of the mutants was slightly reduced. Roughly, all strains exhibit a three-phase growth pattern that might correspond to a latency period followed by an exponential and a stationary phase of growth. The activity of *pel*::*uidA* fusions fell roughly into three classes of response (Fig. 1B,C): *pelA*::*uidA* activity was very limited throughout the growth period; *pelB*::, *pelC*::, and *pelE*::*uidA* fusions were expressed at a moderate level; and a high level of expression was observed with *pelD*::*uidA*. In addition, *pelD*::*uidA* appeared to be induced after only 6 h while the induction of other genes was apparent after 9 h. The *pem*::*uidA* fusion (Fig. 1C) appeared to be moderately expressed from 9 h until 48 h postinoculation. After 48 h, a substantial increase was observed. These results indicate that the different pectinase genes are not regulated in the same manner during the first stage of pathogenesis. Early and significant expression of *pelD*::*uidA*, late induction of *pem*::*uidA*, and the absence of any significant induction of *pelA*::*uidA* are noteworthy observations.

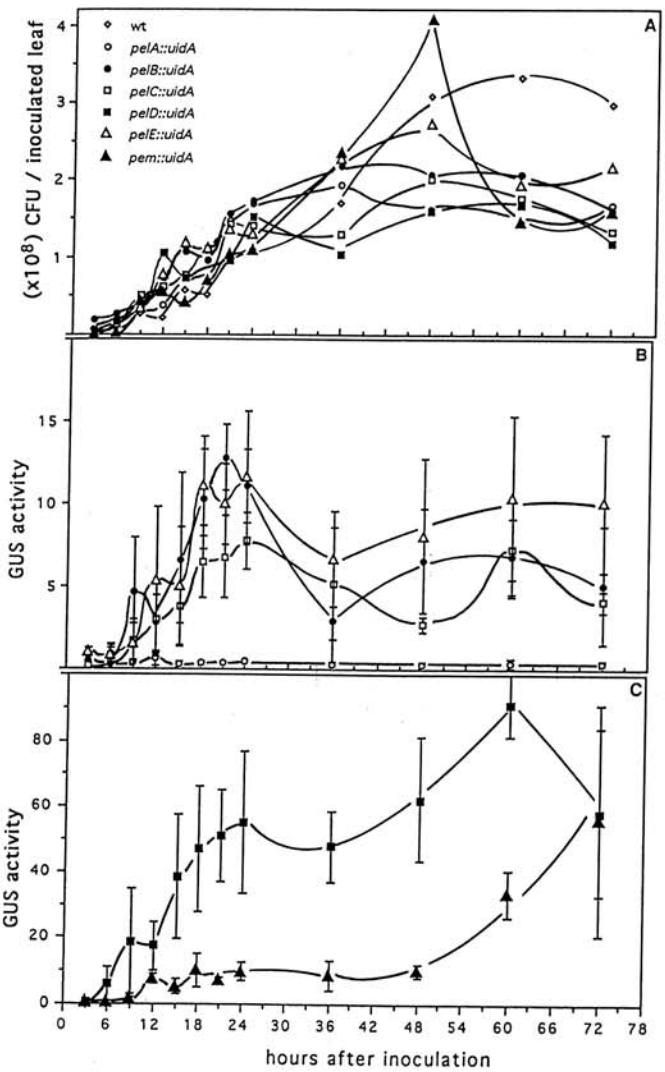

Fig. 1. Time course expression of *pel*:: and *pem*::*uidA* gene fusions in L37 cells, after inoculation into African violets. **(A)** Growth of L37 (wt) and derived mutant cells (*pel*::, *pem*::*uidA*), as indicated on graph, was recorded over 72 h after leaf inoculation (see Materials and Methods). Each point represents the mean of four independent experiments. For legibility, standard deviations are not shown. **(B and C)** GUS specific activity, assayed as specified in Materials and Methods, corresponds to 10^9 CFU. Symbols are as in A. Experiment was performed in triplicate; standard deviations are shown. Y axis scale on graphs is different.

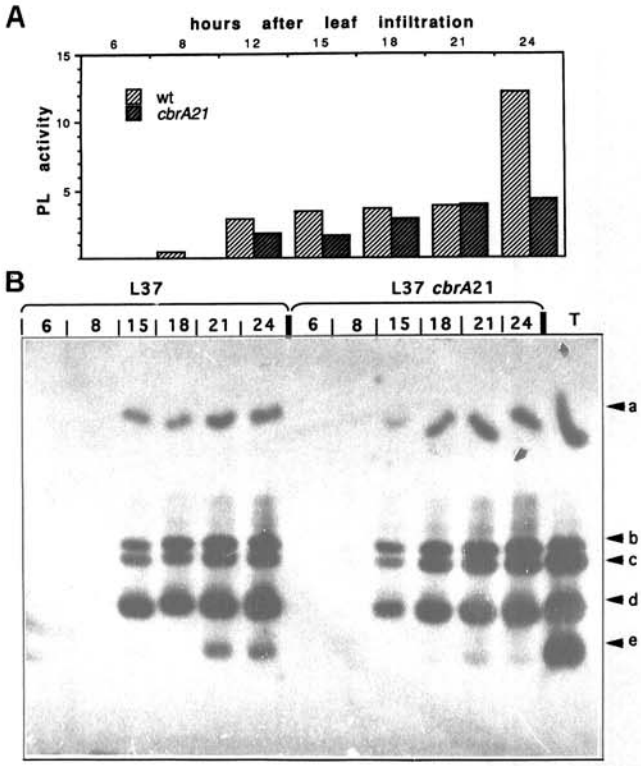

Fig. 2. Time course pectate-lyase (PL) activity recorded in fluids from leaves infiltrated with L37 and L37 *cbrA-21* cells as indicated on each panel. **(A)** Total PL activity, assayed as reported in Materials and Methods, corresponds to 10^9 CFU. Experiment was carried out in duplicate; results were similar. **(B)** Pectate lyase isoenzymes, indicated by arrowheads (lane T: PLa-e detected in a culture supernatant from L37), were separated by electrofocusing on a polyacrylamide gel; their activity was detected using polygalacturonate as a substrate.

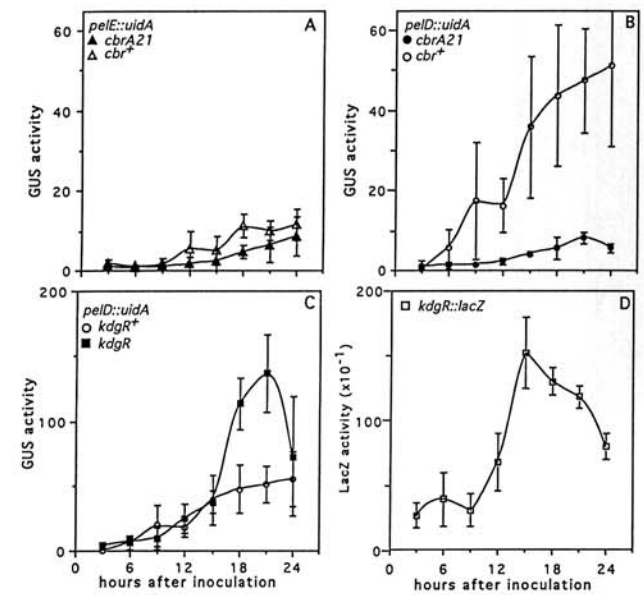

Fig. 3. Time course expression of *pelD*::, *pelE*::*uidA*, and *kdgR*::*lacZ* gene fusions in L37 and derived mutant cells, after inoculation into African violets. Experiments conducted and GUS activity determined as in Figure 1. LacZ activity, expressed in Miller units, corresponds to 10^9 CFU. The *pel* and *kdgR* fusions analyzed, and their genetic backgrounds corresponding to curve symbols, are indicated on each graph **(A, B, C, and D)**. Four independent experiments were performed; standard deviations are shown.

Pels production in L37 and L37 *cbrA-21*, after leaf infiltration.

The production of Pels was previously shown to be stimulated by low iron levels in the wild-type strain but not in a *cbr* mutant (Sauvage and Expert 1994). To investigate whether iron was critical for Pels production during pathogenesis, we examined the possible influence of the *cbrA-21* mutation on global bacterial pectate-lyase (PL) activity produced after leaf infiltration. PL activity in plant fluids was quantitatively and qualitatively assayed during 24 h postinfiltration (Fig. 2). After 6 h, PL activity appeared to increase gradually until 21 h, perhaps more significantly in L37 than in L37 *cbrA-21* cells. At 24 h, a significant increase in activity was observed only in parental cells (Fig. 2A). The absence of any such increase in L37 *cbrA-21* corresponded with a reduction in PelE activity, as visualized on the zymogram (Fig. 2B). A decrease in PelD activity, although not readily visible on the photograph, was also noticed at 15 h in L37 *cbrA-21* cells. This effect is not detected at later times, presumably because the enzyme is present in saturating amounts.

Activity of *pelD*:: and *pelE*::*uidA* gene fusions in mutants L37 *cbrA-21*, L37 *cbsE-1*, and L37 *kdgR*, after leaf inoculation.

In light of the above data, we examined the effect of the *cbrA-21*, *cbsE-1*, and *kdgR* mutations on the transcriptional activity of *pelD* and *pelE*. Experiments were conducted as reported for the parental counterparts.

Figure 3A reveals no major difference in *pelE*::*uidA* activity between L37 and L37 *cbrA-21*. However, the expression level for this fusion was always lower in the mutant than in the wild type. In contrast, *pelD*::*uidA* was not induced in *cbrA-21* cells (Fig. 3B). Therefore, the *pelD* and *pelE* transcriptional patterns are not representative of the variations observed on the zymogram (Fig. 2B). A difference between PelD and PelE specific activity relative to the substrate used for isoenzyme detection, polygalacturonate, may explain this result. Or, possibly, these isoenzymes may differ in their diffusion property or accessibility to the substrate, critical factors in the method used for their detection. The effect of the *kdgR* mutation on *pelE*::*uidA* expression could not be assessed because of the lack of experimental reproducibility. The activity of *pelD*::*uidA* was the same in L37 and L37 *kdgR* cells for 15 h following inoculation, after which a significant increase, with a maximum around 20 h, was observed in L37 *kdgR* only (Fig. 3C). This shows that *pelD*::*uidA*, in the absence of functional KdgR repressor, is inducible in planta. In addition, Figure 3D indicates that the activity of a *kdgR*::*lacZ* fusion increased after 12 h postinoculation, with a maximum after 15 h. This increase could not result from the lack of a functional *kdgR* allele since this gene is not self-regulated (S. Reverchon, personal communication). The *cbrA-21* and *kdgR* mutations did not influence the bacterial growth rate (data not shown).

In the presence of the *cbsE-1* mutation, which disrupts biosynthesis of chrysobactin, *pelD*::*uidA* activity also was reduced (data not shown). This effect was correlated with the poor growth of the mutant strain in planta. Indeed, after 24 h postinoculation, 10^6 CFU per leaf were recovered with L37 *pelD*::*uidA cbsE-1*, versus 5×10^9 CFU with L37 *pelD*::*uidA cbrA-21*. These results suggest the existence of a link between

the potential of the bacterium to acquire iron via chrysobactin and *pelD* expression in planta.

Activity of *pelD*::*uidA* gene fusion in L37 and L37 *cbrA-21*, grown in the presence of an Fe(III) chelator.

Sauvage and Expert (1994) reported that *pelB*::, *pelC*::, and *pelE*::*uidA* activities, unlike *pelD*::*uidA*, were stimulated in bacteria grown in M63 medium made poor in iron by a treatment with 8-hydroxyquinoline. In addition, the stimulatory effect of low iron levels on *pelB*::, *pelC*::, and *pelE*::*uidA* was abolished in a *cbr* mutant; *pelD*::*uidA* activity was even lower than the iron-repressed level in parental cells. It is noteworthy that M63 medium treated with 8-hydroxyquinoline still contains traces of iron, probably due to the presence of high levels of phosphate (Mahé et al. 1995). Considering the data observed in planta we decided to revise our conditions of iron depletion. Iron inaccessibility was achieved by addition of EDDA (log FeL = 34) to Tris medium containing only 2 mM phosphate. The activity of *pelD*::*uidA* was recorded in wild-type and *cbrA-21* cells during their growth in Tris medium supplemented with $FeCl_3$ or with EDDA (Fig. 4). The presence of $FeCl_3$ appeared to decrease *pelD*::*uidA* activity and increase the growth rate in both strains (Fig. 4). Interestingly, deferration of Tris medium with Chelex, an iron-binding resin that can remove iron from relatively weak ligands (log FeL ≤ 12), had no effect on expression of the fusion (data not shown). This led us to assume that either traces of iron were still present in the Tris medium after Chelex treatment or they were possibly present following cell lysis in the culture. From these data, we concluded that the activity of *pelD*::*uidA* increases only in severe conditions of iron starvation, i.e., when iron-scavenging agents are present. The low level of expression of *pelD*::*uidA* in a *cbr* mutant relative to the parental strain, observed by Sauvage and Expert (1994), can thus be explained by the fact that the mutant is derepressed for production of chrysobactin and hence is less susceptible to iron deprivation.

Activity of *pelD*::*uidA* gene fusion in L37 and L37 *cbrA-21*, *cbsE-1* single or double mutants grown in leaf IF.

Leaf IF harvested from healthy plants reflects the environmental conditions that may be encountered by bacterial cells during pathogenesis (Neema et al. 1993). Interestingly, a *lacZ* fusion to the chrysobactin operon (*fct*::*lacZ9*) is induced when bacterial cells are grown in IF; induction is prevented by addition of $FeCl_3$ in the culture (Masclaux and Expert 1995). To examine whether the expression of *pelD*:: relative to *pelE*::*uidA* was influenced by the iron status in IF, *pelD*::*uidA* activity was recorded in bacterial cells grown under such conditions. Activity of *pelD*::*uidA* was analyzed in wild-type, *cbrA-21*, and *cbsE-1* backgrounds (Table 1).

In parental, as in *cbrA-21* cells, *pelD*::*uidA* expression in IF decreased when supplemented with $FeCl_3$. In *cbsE-1*, as in *cbsE-1 cbrA-21* cells, expression of *pelD*::*uidA* was higher than in parental cells and addition of $FeCl_3$ into IF was ineffective. These data show that disruption of chrysobactin biosynthesis enhances the expression of *pelD*::*uidA* while inactivation of iron transport mediated by achromobactin has no influence. In addition, the lack of repression of the fusion by iron in chrysobactin-deficient cells indicates that the iron supplied cannot be internalized via the achromobactin dependent pathway. Hence, IF must contain strong iron-free ligands scavenging the Fe(III) supplied.

In parental cells, *pelD*::*uidA* expression in IF treated with Chelex (CIF) was higher than in IF, showing that deferration stimulates *pelD* gene transcriptional activity. In *cbsE-1* cells, *pelD*::*uidA* expression in CIF was as in IF, but in CIF the addition of iron reduced the expression. This indicates that the treatment with Chelex changes IF composition. Indeed, it is possible that the resin also removes other metal cations, thus resulting in the exposure of potential iron ligands. Iron bound to these compounds could be used by bacterial cells in the absence of a functional chrysobactin- or achromobactin-mediated pathway. In chrysobactin-proficient cells, repression of *pelD*::*uidA* by iron was less efficient in CIF than in IF, which would suggest the occurrence of weak iron-free ligands in CIF. These data demonstrate that *pelD*::*uidA* responds specifically to variations in the iron status of leaf IF fluid.

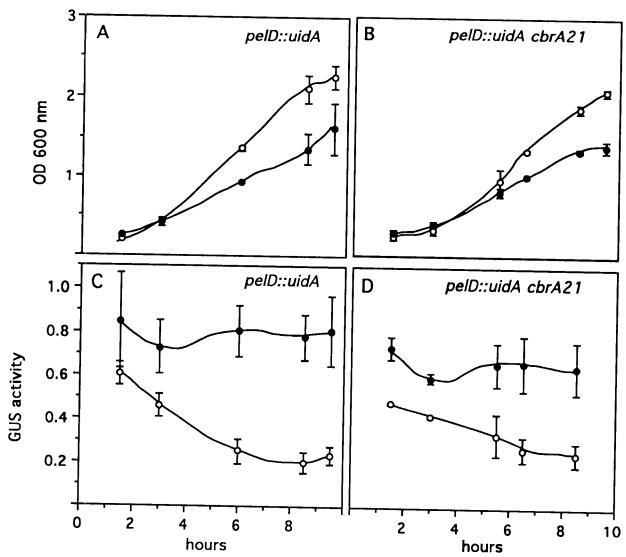

Fig. 4. Expression of *pelD*::*uidA* gene fusion in L37 and L37 *cbrA-21* cells grown in Tris medium supplemented with $FeCl_3$ (open circles) or with EDDA (ethylene-diamine-*N*,*N*′-bis(2-(hydroxyphenyl))acetic acid) (solid circles). Strain genotypes are indicated on each graph. **(A and B)** Bacterial growth. **(C and D)** GUS activity, determined as described in Materials and Methods, is expressed per optical density unit (about 10^9 CFU/ml). Experiment carried out in duplicate; standard deviations are shown.

Table 1. Expression of *pelD*:: and *pelE*::*uidA* gene fusions in L37 and derived mutant cells grown in leaf intercellular fluid (IF)[a]

Genotype	IF	IF +Fe	CIF	CIF +Fe
pelD::*uidA*	0.500	0.270	0.730	0.460
pelD::*uidA cbrA-21*	0.544	0.273	ND[b]	ND[b]
pelD::*uidA cbsE-1*	1.000	0.900	0.850	0.400
pelD::*uidA cbsE-1 cbrA-21*	0.970	1.090	0.992	0.392
pelE::*uidA*	0.460	0.480	0.800	0.500
Optical density at 600 nm	1.5	1.5	1	1

[a] GUS activity (per optical density unit, about 10^9 CFU/ml) was recorded in cultures in IF and in Chelex-treated IF (CIF), supplemented or not with $FeCl_3$(+ Fe), reaching an optical density at 600 nm as indicated. Details are in Material and Methods and elsewhere in the text. Experiment was performed in duplicate with different batches of IF; results were similar.

[b] Not determined.

This response appears to depend on an intracellular iron pool that is depleted in the absence of a functional chrysobactin iron transport pathway.

DISCUSSION

To elucidate whether the regulatory effect of iron on *pel* gene expression observed under laboratory conditions had physiological significance in soft rot caused by *E. chrysanthemi* strain 3937 on African violets, we analyzed the expression in planta of the different *pel* and *pem* genes important for virulence.

Regarding the prevalence of pectinolytic enzymes in soft rot symptoms, it is noteworthy that the experimental model developed on African violets stresses the dynamic aspect of the disease and illustrates a number of points that have long been questioned. It is noteworthy that the transcriptional expression of a *pel* gene does not correlate with the importance of its product in virulence: while *pelE*:: and, more significantly, *pelA*::*uidA* seem to be weakly expressed relative to *pelD*::*uidA* on African violets, no difference with regard to symptom reduction was noted between the mutants (Boccara et al. 1988). An important point would be to determine the specific activity of each isoenzyme relative to pectic substrates, the latter having yet been poorly characterized. Interesting also is the second level of induction of the *pem*::*uidA* fusion, which is high relative to the first one and late relative to *pel* genes induction. This was unexpected because demethylation of pectin by PME is assumed to be essential for Pels to proceed. Thus, one can question the degree of methylation that Pels may tolerate while still hydrolyzing pectic compounds.

With regard to the role of iron, our data provide a new viewpoint on how a pathogen regulates its virulence and responds to the plant environment. Several lines of evidence indicate that iron availability in the host plant controls *pel* gene expression. The sequential pattern observed on African violets for *pel* gene induction prompted us to investigate the effect of the *cbrA-21* mutation on global PL activity produced by bacteria after inoculation. Production of Pels was reduced in L37 *cbrA-21* in a similar fashion as in a culture grown under conditions of iron limitation. Considering the pleiotropic effect of the *cbrA-21* mutation, this effect may be explained quite simply. Achromobactin-mediated iron uptake is affected in the mutant L37 *cbrA-21*. Because this mutation results in derepression of the chrysobactin-mediated iron transport pathway, the mutant is probably less susceptible to iron deprivation than wild-type cells are when entering the host. This resistance to iron deprivation results in a delay in Pels production thus leading to delayed symptoms, as reported by Sauvage and Expert (1994). The delay in the symptomatic phase of the disease caused by this particular mutation indicates that iron is likely to be one of the first signals received by the bacterium to synthesize Pels and to develop symptoms.

We then examined the effect of the *cbrA-21* mutation on *pel* gene transcriptional activity in planta. Whereas *pelE*::*uidA* expression was previously found to be stimulated in the absence of $FeCl_3$ and repressed in a *cbr* mutant (Sauvage and Expert 1994), *pelE*::*uidA* activity in L37 *cbrA-21* was not significantly reduced in planta; however, production of PelE in this mutant appeared to be delayed. In contrast, *pelD*::*uidA* expression was not influenced by $FeCl_3$ levels (Sauvage and Expert 1994), although it proved to be highly reduced in L37 *cbrA-21* in planta and responded to iron limitation caused by iron-scavenging agents. It is also significant that the expression of *pelD*::*uidA* was influenced by a mutation affecting the production of chrysobactin. Indeed, in a chrysobactin-deficient mutant no *pelD*::*uidA* expression was observed in planta, probably because of the poor growth of mutant cells. However, in IF collected from healthy plants, growth of this mutant was not severely affected and *pelD*::*uidA* was significantly expressed. This shows that iron is not as readily accessible to the bacterium during pathogenesis as it is in IFs from healthy plants. In addition, since *pelD* gene expression is stimulated under severe iron limitation it is not surprising that L37 *cbsE-1*, particularly susceptible to iron deprivation because it is unable to produce chrysobactin, expresses *pelD*::*uidA* at higher levels. The question is how to interpret these data regarding pathogenesis. In our model, *pelD* is the first *pel* gene to be induced. Its expression precedes induction of the chrysobactin *fct cbsCEBA* operon. Wild-type cells produce chrysobactin when they are starved intracellularly for iron. Thus, induction of *pelD*::*uidA* coincides with the phase during which bacteria become iron-starved. In contrast, *pelE*::, *pelB*::, and *pelC*::*uidA*, which are moderately sensitive to iron limitation, appear to be induced later, presumably when the intracellular iron pool is not completely depleted because of the production of chrysobactin. Therefore, we are tempted to propose that iron not only induces *pel* gene expression but also imposes a temporal pattern on *pel* induction. In this context, the poor growth of the mutant affected both in PelD and chrysobactin production, relative to a PelD (this study) or a chrysobactin (Masclaux and Expert 1995) single mutant, suggests that the hydrolytic activity of PelD initiates maceration and, hence, the release of diverse iron substrates from damaged plant cells. The regulatory effect on *pelD*::*uidA*, detected in IFs following variations in iron status and in relation to the chrysobactin phenotype, illustrates the complexity of the scenario and the accuracy of the underlying sensory system. A homologue of the Fur repressor, which mediates a coordinated expression of iron regulated functions in various bacterial species (Litwin and Calderwood 1993), is probably not the only regulator prevailing in our model.

Because KdgR plays a central role in the regulation of pectinolysis, we looked at the effect of a *kdgR* mutation on *pelD*::*uidA* expression in planta. The activity of *pelD*::*uidA* was not influenced by a *kdgR* mutation during the first 15 h following inoculation, thus suggesting the absence of repression of the fusion via KdgR in the wild-type strain within this period of time. Thus, one may consider that pectic inducers are released at the onset of infection and are maintained at a level at which the *pelD* gene is derepressed. However, the expression of *kdgR*::*lacZ* in planta indicates that KdgR is produced. After 15 h, *pelD*::*uidA* was still inducible in L37 *kdgR*, indicating that the KdgR repressor is more active during this second phase in L37. Interestingly, this phase correlates with the maximum level of expression observed for *kdgR*::*lacZ*. In this case, it is possible that in L37 the concentration of pectic oligomers is too low to mediate total inactivation of the repressor. Therefore, it seems that derepression of *pelD*::*uidA* via inactivation of KdgR is not critical early after inoculation. Furthermore, one wonders whether derepression of *pel* genes by pectic inducers is significant in terms of exacerbation of the maceration process since transcriptional expression of

kdgR increases after 9 h, when pectic oligomers have been released. In contrast, the effect of the *cbrA-21* mutation is apparent 6 h postinoculation. It is possible that the regulatory effect mediated by KdgR, which is very efficient in terms of gene derepression levels, is better adapted to a saprophytic life than to the pathogenic cycle observed on African violets. The message emerging from this analysis is that in vivo studies are required to elucidate the fine mechanisms underlying microbial pathogenicity.

Bauer et al. (1994) have recently shown that *E. chrysanthemi* can trigger a hypersensitive response (HR) on tobacco leaves when the production of Pels is disrupted. This behavior has been assigned to the presence of a *hrp* locus. The *hrp* locus is not absolutely required for *E. chrysanthemi* pathogenicity in chicory leaves, but Hrp$^-$ mutants are quantitatively reduced in their ability to incite pathogenesis (Bauer et al. 1994). Unlike typical phytopathogens capable of inducing HR, *E. chrysanthemi* has a broad host range and does not display the basic characteristics of compatible and incompatible interactions. The ability of *E. chrysanthemi* to infect a large number of hosts is probably related to the performance of its pectinolytic equipment. Indeed, a rapid destruction of plant tissues should allow the bacteria to overcome the host defenses and be independent, in some way, of the *hrp* locus. The existence of a fine tuning to ensure a balanced production of Pels during the infection process should be viewed as a selective advantage for the pathogen. Iron sensing demonstrates this point.

MATERIALS AND METHODS

Plant, bacterial strains, and culture media.

Two-month-old potted plants (*Saintpaulia ionantha,* Blue Rhapsody) were used. The bacterial strains and bacteriophage used are described in Table 2. The rich medium was L broth (Miller 1972). Tris medium (Franza et al. 1991) was used to provide iron-limited conditions. This medium was supplemented with EDDA (ethylene-diamine-*N,N'*-bis(2-(hydroxyphenyl))acetic acid) at a final concentration of 100 μg/ ml to chelate contaminating iron. For iron-replete conditions, $FeCl_3$ was supplied at a final concentration of 20 μM. IF from healthy plants was prepared as described by Neema et al. (1993). For deferration, IF was treated with Chelex 100 resin (H form, Bio-Rad SA, Ivry Sur Seine, France). The resin was presoaked in distilled water and added to IF to give a final concentration of 20% (wt/vol). Then the mixture was shaken for 5 min and filtered through Millipore HA filters (pore size 0.45 μm) for elimination of the resin and sterilization. Glycerol (2 g per liter) and polygalacturonate (4 g per liter) were used as a carbon source and a Pel inducer, respectively.

Construction of bacterial strains.

The *pel::uidA, pem::uidA,* and *kdgR::lacZ* fusions were transduced in strain L37 with phage PhiEC2 (Résibois et al. 1984), as previously described (Franza et al. 1991), using kanamycin (Km) resistance as a selective marker. The phenotype of recipient strains for the production of Pel isoenzymes was determined by electrofocusing in a polyacrylamide gel (Bertheau et al. 1984). The *cbrA-21* and *cbsE-1* mutations were introduced by transduction, using chloramphenicol and streptomycin as selective markers, respectively. The Cbr$^-$ phenotype was checked on CAS agar medium (Schwyn and Neilands 1987), as indicated previously (Expert et al. 1992). The Cbs$^-$ phenotype was indicated by the lack of catechol production, as assayed by the method of Arnow (1937).

Determination of β-galactosidase and β-glucuronidase activities in bacteria grown in planta.

Enzyme activities were determined in bacterial cells collected from inoculated plant leaves as previously described

Table 2. Bacterial strains and phage used in this study

Strain	Characteristics	References or source
Erwinia chrysanthemi		
3937 *pelA::uidA*	*pelA,* Km^r	Sauvage and Expert 1994
3937 *pelB::uidA*	*pelB,* Km^r	Sauvage and Expert 1994
3937 *pelC::uidA*	*pelC,* Km^r	Sauvage and Expert 1994
3937 *pelD::uidA*	*pelD,* Km^r	Sauvage and Expert 1994
3937 *pelE::uidA*	*pelE,* Km^r	Sauvage and Expert 1994
3937 *cbsE-1*	*cbs*: with an Ω interposon inserted at the *Bam*HI site of the *fct cbsCEBA* operon, Spec^r, Sm^r	Mahé et al. 1995
A1077	*lmrt* (Con) *lacZ kdgR*::Mud*IPR13,* Cm^r	Hugouvieux-Cotte-Pattat et al. 1992
A1789	*pem::uidA,* Km^r	Hugouvieux-Cotte-Pattat et al. 1992
L37	Lac$^-$ derivative from 3937	Hugouvieux-Cotte-Pattat and Robert-Baudouy 1985
L37 *cbrA-21*	*cbrA-21*: *cbrA*::Mud*IIPR13,* Cm^r	Mahé et al. 1995
L37 *pelA::uidA*	*pelA,* Km^r	This work
L37 *pelB::uidA*	*pelB,* Km^r	This work
L37 *pelC::uidA*	*pelC,* Km^r	This work
L37 *pelD::uidA*	*peD,* Km^r	This work
L37 *pelE::uidA*	*peE,* Km^r	This work
L37 *kdgR::lacZ*	*kdgR,* Cm^r	This work
L37 *pelD::uidA cbrA-21*	*pelD, cbr,* Km^r, Cm^r	This work
L37 *pelD::uidA cbsE-1*	*pelD, cbs,* Km^r, Sm^r, Spec^r	This work
L37 *pelD::uidA cbsE-1 cbrA-21*	*pelD, cbs, cbr,* Km^r, Sm^r, Spec^r, and Cm^r	This work
L37 *pelD::uidA kdgR*	*pelD, kdgR::lacZ,* Km^r, Cm^r	This work
L37 *pelE::uidA cbrA-21*	*pelE, cbr,* Km^r, Cm^r	This work
L37 *pelE::uidA kdgR*	*pelE, kdgR::lacZ,* Km^r, Cm^r	This work
Phage		
PhiEC2	General transducing phage of *E. chrysanthemi*	Résibois et al. 1984

(Masclaux and Expert 1995). For each strain, four to six independent experiments were carried out. The β-galactosidase activity (LacZ activity) was assayed by the method of Miller (1972) with minor modifications as described by Masclaux and Expert (1995). β-glucuronidase activity (GUS activity) was assayed as described by Sauvage and Expert (1994), except that specific activity was expressed in nanomoles of paranitrophenol liberated per minute per 10^9 CFU. Concurrent enumeration of the bacterial populations (CFU) present in IF was determined on agar medium containing the appropriate antibiotics (Masclaux and Expert 1995).

Determination of PL activity in isolated leaves from potted plants.

Plant leaves were infiltrated in vacuo with an inoculum consisting of a bacterial culture grown in iron-supplemented Tris medium reaching an optical density at 600 nm of 0.4. Infiltrated leaves were then placed in petri dishes on a 5-mm-high-glass-balls bed soaked in sterile water to maintain moisture. Dishes were incubated at 30°C. Intercellular fluids were collected over time, and bacterial populations were counted. The samples were then centrifuged at 5,000 rpm in a microfuge (Medical Scientific Equipment, Leicester, UK) to eliminate bacterial cells. PL activity was determined in filter-sterilized supernatant fluids as reported by Sauvage and Expert (1994), with a minor modification: one unit of PL was defined as 1 μM unsaturated product liberated per minute per 10^9 CFU. Pels isoenzymes were analyzed by electrofocusing in an ultrathin polyacrylamide gel. Their activity was detected after transfer by blotting the gel on a substrate-containing agar gel as described by Bertheau et al. (1984). Equal samples corresponding to 10^8 CFU were loaded.

Determination of β-glucuronidase activity in bacterial cultures.

For iron replete and deplete conditions, an inoculum from an overnight bacterial culture in L broth was diluted in fresh Tris medium, supplemented with $FeCl_3$ or EDDA, to give an optical density at 600 nm of 0.1. IF was inoculated with an 8-h iron-supplemented culture in Tris medium to give an optical density at 600 nm of 0.002. Cultures were grown aerobically. Samples were collected and immediately frozen. β-glucuronidase activity was assayed as reported above except that activity was expressed in nanomoles of paranitrophenol liberated per minute per optical density at 600 nm.

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