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Production of *Erwinia chrysanthemi* pectinases in potato tubers showing high or low level of resistance to soft-rot

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Abstract

Erwinia chrysanthemi was used for inoculation of two lines of potato tubers with different degrees of resistance to soft-rot: cultivar Katahdin, a very sensitive material, and the somatic hybrid of *Solanum brevidens* and *Solanum tuberosum* as a resistant plant. Infection was performed with the *E. chrysanthemi* wild-type strain 3937 and various mutants containing a transcriptional fusion of one pectinase gene *(pem, pelA* or *pelE)*, together with the reporter gene *gus*. Compared with the susceptible tubers, the accumulation of pectate lyase in the resistant tubers is limited. This limitation of the pectate lyase production seems to take place at the transcriptional level, with a striking decrease of the expression of the *pelA::gus* fusion and a decrease in the first steps of infection of the *pelE::gus* fusion expression. The pectinase genes appeared to be differentially regulated in the two potato lines. This study also revealed the major role of pectin methyl esterase in the infection process which occurs in potato tubers resistant to soft-rot. Indeed, the maceration of somatic hybrid tubers by the *pem* mutant was strongly reduced in comparison with the wild-type. We suggest that the resistant tubers have the advantage of a high methylated pectin which is difficult to degrade by the *E. chrysanthemi* pectinases.

Introduction

The enterobacterium *Erwinia chrysanthemi* causes soft-rot disease of various plants. This pathogen produces a battery of depolymerizing enzymes, including several pectinases that play a major role in the maceration of plant tissues (Collmer and Keen, 1986; Barras et al., 1994). Pectin, a galacturonic acid containing polysaccharide, is one of the main components of the primary cell wall and middle lamella of plant cells. To depolymerize and catabolize pectin, *E. chrysanthemi* produces a series of enzymes. Pectin methyl esterase (PME, encoded by the *pem* gene) removes the methoxyl groups from pectin. Then pectate lyases (PL, encoded by the *pel* genes) cleave internal glycosidic linkages in pectic polymers by β-elimination. Most *E. chrysanthemi* strains secrete five major PL isoenzymes which differ in their isoelectric points: acidic for PLa, neutral for PLb and PLc and basic for PLd and PLe (Bertheau et al., 1984; Boccara et al., 1992). The five *pel* genes and the *pem* gene are organized in two clusters *-pelB, pelC* and *pelA, pelE, pelD, pem-*, widely separated on the bacterial chromosome (Hugouvieux-Cotte-Pattat et al., 1989). However, *pem* and each *pel* gene constitute independent transcriptional units and can respond to different transcriptional controls (Kotoujansky, 1987).

Various environmental stimuli influence the production of the *E. chrysanthemi* pectinases. Construction of gene fusions with the reporter gene *gus* (or *uidA*) permitted us to follow individually the expression of each pectinase gene (Hugouvieux-Cotte-Pattat et al., 1992). In synthetic culture media, transcription of the *pelA, pelB, pelC, pelD, pelE* and

pem genes is induced by pectin catabolic products and by the late exponential growth phase and is repressed in conditions of catabolite repression, nitrogen starvation and at a high temperature. Expression of the genes *pelA, pelD* and *pelE* is generally very sensitive to these conditions and is also modulated by anaerobiosis (Hugouvieux-Cotte-Pattat et al., 1992). Increasing osmolarity of the medium gave different responses depending on the gene; it increased *pelE*, decreased *pelD* but did not affect *pelA, pelB, pelC* or *pem* expression (Hugouvieux-Cotte-Pattat et al., 1992). Transcription of the *pelB, pelC* and *pelE* genes is also stimulated under low iron conditions (Sauvage and Expert, 1994). The regulation of these genes is then subjected to a panoply of signals and requires several regulatory systems. These regulatory systems are poorly characterized, except for the system involved in induction by pectic derivatives, mediated by the KdgR repressor (Nasser et al., 1994).

E. chrysanthemi can attack many plants and the different pectinases contribute to its wide host range since the role of each pectinase in pathogenesis is dependent on the host (Beaulieu et al., 1993). The amount of each PL present in macerated tissue varies between host plants (Beaulieu et al., 1993) or between specific tissues (Lojkowska et al., 1993). The role of the individual *pel* genes in pathogenicity was first analysed by infection of *Saintpaulia ionantha* with the *E. chrysanthemi* mutants deficient in the synthesis of one isoenzyme; in this system, inactivation of *pelA, pelD, pelE* and *pem* genes significantly reduced virulence (Boccara et al., 1988; Boccara and Chatain, 1989). Inoculation of potato with the mutants containing *gus* fusions in each pectinase gene revealed that *pelA* is very highly expressed in tubers and likewise *pelE* in plants, compared with their expression in synthetic media (Lojkowska et al., 1993). Plant factors may modulate the expression of these genes (Bourson et al., 1993), which appear to be important for virulence in most host plants tested (Boccara et al., 1988; Beaulieu et al., 1993). Studies on potato tubers revealed the important role of the *pem* gene in this plant species, while *pel* genes seemed to be interchangeable (Beaulieu et al., 1993).

Tubers of potato cultivars vary in their relative susceptibility to soft-rot caused by *Erwinia*, but none of the common cultivars are considered to be highly resistant. Some wild *Solanum* species were reported to be resistant to soft-rot (Lojkowska and Kelman, 1989). Also some somatic hybrids, obtained by protoplast fusion between *Solanum brevidens* and *Solanum tuberosum*, were found to present a high level of resistance to soft-rot (Austin et al., 1988; Lojkowska and Kelman, 1994). The purpose of our study was to analyse the production of *E. chrysanthemi* pectinases in potato tubers showing a high or low level of resistance to soft rot.

Materials and methods

Bacterial strains and growth conditions. The *E. chrysanthemi* strains used in this study were the wild-type strain 3937 and a set of the derivative mutants with transcriptional fusions of three pectinase genes, together with the reporter gene encoding *gus* (or *uidA*): A1888 (*pelA::gus*), A1881 (*pelE::gus*) and A1789 (*pem::gus*). Each construction was recombined into the bacterial chromosome in place of the wild-type gene (Hugouvieux-Cotte-Pattat et al., 1992). Bacteria were grown in M63 synthetic medium with glycerol as a carbon source. Liquid cultures were grown in a shaking incubator for 24 hours at 30 °C, and were then washed with M63 and adjusted to 2 x 10^9 colony forming units (cfu) per ml.

Plant material. Potato tubers of *S. tuberosum* L., cultivar Katahdin and the somatic hybrid 946 of *S. brevidens* and *S. tuberosum* (Austin et al., 1988), were grown in the greenhouse of the Institute for Potato Research, Bonin, Poland.

Pathogenicity tests. Tubers were washed and immersed twice for 20 min in 5% sodium hypochlorite (Javel), rinsed in sterile deionized water and air-dried under a laminar flow hood. Sterile polypropylene pipet tips containing 50 μl of bacterial suspension (2 $\times$ 10^9 cfu/ml) were inserted, in a randomized manner, into the tuber parenchyma to a depth of 10 mm (Maher and Kelman, 1983; Austin et al., 1988). Usually 4 pipet tips were used per tuber: 3 contained suspensions of different mutants and the 4th contained the wild-type strain. Five tubers were inoculated with each mutant and incubated at 30 °C in a dew chamber at 100% relative humidity. Disease severity was determined every day for 3 days following inoculation. Tubers were sliced vertically through each inoculation point. The weight of decayed tissue was determined and taken as the characteristic of disease severity. Estimation of the multiplication of bacteria, the PL and the GUS activities were performed as described below.

Bacterial multiplication in tuber tissue. For the determination of bacterial multiplication, samples of rotted tissue from five tubers were collected and homogenized in M63. Colony forming units (cfu) were determined by plating appropriate dilutions on agar plates with or without kanamycin (20 μg/ml).

Enzyme assays. Assays of PL and GUS were performed on toluenized extracts of infected tissues collected from five tubers. PL activity was determined by the degradation of PGA to unsaturated products that absorb at 235 nm (Moran et al., 1968). One unit of PL activity is the amount of enzyme able to liberate one μmol of unsaturated products per min. Specific activity of PL is expressed in units per mg of bacterial dry weight. GUS activity was measured by the degradation of *p*-nitrophenyl-β-D-glucuronide into *p*-nitrophenol that absorbs at 405nm (Bardonnet and Blanco, 1992). One unit of GUS activity is the amount of enzyme able to liberate one nmol of product per min. Specific activity of GUS is expressed as units per mg of bacterial dry weight. The total PL or GUS activity per inoculation site was obtained by multiplication of the PL activity per g of tissue by the weight of rotted tissue.

Statistical analysis. The influence of the time and of the potato cultivar on several variables (rot, PL and GUS activities) was tested with the two factor variance analysis, performed using the StatviewII^{TM} software.

Table 1. Rot progression in susceptible and resistant tubers. Potato tubers (Katahdin and somatic hybrid) were inoculated with the *E. chrysanthemi* wild-type strain using sterile pipet tips containing 50 μl of bacterial suspension (2×10^9 cfu/ml) and incubated in a dew chamber at 30 °C. Weight of decayed tissue was determined during the 3 days following inoculation. The mean value and the standard deviation of 3 independent experiments are given. The two factor variance analysis performed indicates that the weight of rotted tissue is significantly higher on the susceptible potato line ($p = 0.0004$)

Time	Katahdin, gram of rotted tissue	Somatic hybrid, gram of rotted tissue
24 h	0.87 ± 0.09	0.84 ± 0.03
48 h	5.80 ± 0.60	5.00 ± 0.20
72 h	13.70 ± 1.70	7.00 ± 0.20

Table 2. Kinetics of bacterial multiplication on susceptible and resistant potato tubers. Katahdin and somatic hybrid tubers were inoculated with the *E. chrysanthemi* wild-type strain 3937. Bacteria were isolated from the rotted tissue and dilution plating was performed each day to determine the number of cfu/g of rotted tissue.The total number of cfu at the inoculation site was obtained by multiplication of the cfu/g of tissue by the weight of rotted tissue. The mean value and the standard deviation of 3 independent experiments are given. The two factor variance analysis performed indicates that the number of bacteria is not significantly higher on the susceptible potato line ($p = 0.11$). Therefore, all the subsequent enzymatic specific activities were calculated with the mean value shown in this table

Time	Katahdin cfu/ inoculation site	Somatic hybrid cfu/ inoculation site	Mean value
24 h	2.8 ± 0.8	2.0 ± 0.9	2.4
48 h	20.5 ± 4.4	18.9 ± 5.5	19.7
72 h	99.3 ± 53.0	44.8 ± 22.0	72.1

Results

Disease severity and bacterial growth on potato tubers. Three independent experiments were set up to analyze the infection process in tubers of the two lines of potato: the susceptible cultivar Katahdin and the resistant somatic hybrid of *S. brevidens* and *S. tuberosum*. The rot progress was significantly lower in tubers of the somatic hybrid than in tubers of Katahdin when inoculations were performed with the wild-type *E. chrysanthemi* strain (Table 1).

The growth of the *E. chrysanthemi* population appears slightly less active in the somatic hybrid than in the Katahdin tubers, but without statistical significance (Table 2).

Pectate lyase production on susceptible and resistant potato tubers. The PL activity was assayed after inoculation of the two lines of tubers. We followed (i) the specific activity (unit of enzyme per mg of bacterial dry weight) that represents the amount of active enzyme produced by a given number of bacteria and (ii) the total PL activity present in the whole infected area (unit of enzyme per inoculation site) resulting from the PL production by the entire population of bacteria.

Similar kinetics of PL specific activity were observed on both potato lines (Table 3A): the strong increase occurring during the first day was followed by a decrease in the PL specific activity. Similar patterns of the PL kinetics after inoculation with *E. chrysanthemi* were found in the different lines of tubers, Katahdin and somatic hybrid (this paper) and Mona Lisa (Lojkowska et al., 1993). The major difference between the resistant and susceptible potato lines is that the PL specific activity appears to be significantly lower in the tissue of resistant tubers (Table 3A).

The same qualitative profile was observed both in susceptible and resistant tubers, but with a higher

Table 3. Pectate-lyase activity after inoculation of resistant and susceptible potato tubers. Katahdin and somatic hybrid tubers were inoculated with the wild-type strain, as described in Table 1. PL activity was determined each day. The mean value and the standard deviation of 3 independent experiments are given. **A**, Specific activity of PL is expressed as enzyme units per mg of bacterial dry weight (BDW). The two factor variance analysis performed indicates that the PL specific activity is significantly higher on susceptible tubers ($p = 0.002$). **B**, Total PL activity detected in the rotted tissue is expressed as enzyme units per inoculation site. The two factor variance analysis performed indicates that the total PL activity per inoculation site is significantly higher on susceptible tubers ($p = 0.01$)

A		
Time	Katahdin PL/mg BDW	Somatic hybrid PL/mg BDW
24 h	50.9 ± 16.0	22.6 ± 9.8
48 h	45.6 ± 15.5	13.4 ± 1.3
72 h	4.0 ± 2.8	1.4 ± 0.4

B		
Time	Katahdin PL/inoculation site	Somatic hybrid PL/inoculation site
24 h	11.0 ± 0.3	3.8 ± 1.9
48 h	50.0 ± 25	19.0 ± 14.0
72 h	77.0 ± 6.0	41.2 ± 3.0

level of the total PL activity in the susceptible tubers. Therefore, the production of PL is limited in the somatic hybrid tubers. Although the PL specific activity decreases after the peak of the first day post-inoculation, the total PL activity and the rot continue to progress in the tubers (Table 1, Table 3B). The PL accumulation resulting from an initial burst (first day) and then from residual production (Table 3A) suggests that the PLs are quite stable in the tubers. The total PL activity per inoculation site appeared to be clearly related to the rot extent. This result is consistent with the fact that PLs are directly responsible for the maceration caused by *E. chrysanthemi*.

Maceration of the potato tubers by the pelA, pelE *or* pem *mutants.* Several *Erwinia* strains harboring a gene fusion between a pectinase gene and the reporter gene *gus* were used. Among the five isoenzymes of pectate lyase we chose to study the gene *pelA*, which is supposed to play a key role in virulence, and *pelE*, because its product is quantitatively the major pectate lyase. On both lines of potato, a significant decrease of the rotting was observed for the *pelE* mutant while

Table 4. Maceration observed in resistant and susceptible tubers 3 days after inoculation with the *E. chrysanthemi* wild-type and the mutant strains. The mean value and the standard deviation of 3 independent experiments are given

	Katahdin, gram of rotted tissue	% [a]	Somatic hybrid, gram of rotted tissue	% [a]
Wild-type	12.9 ± 1.5	100	7.1 ± 0.4	100
pelA :gus	10.4 ± 3.5	81	6.2 ± 2.5	87
pelE::gus	8.3 ± 0.6	64	4.3 ± 2.9	61
pem::gus	10.0 ± 0.4	78	2.3 ± 2.2	32

[a] percentage of maceration by comparison with the wild-type.

the *pelA* mutant was only slightly affected as regards its maceration ability (Table 4). To study the importance of the PME in *Erwinia* pathogenicity, the strain harboring a gene fusion between the *pem* gene and the reporter gene *gus* was used. With the *pem* mutant, we recorded 78% of the wild-type maceration in Katahdin tubers but only 32% of the wild-type maceration in somatic hybrid tubers (Table 4). The mutation of the *pem* gene strongly affects the virulence of *E. chrysanthemi* in somatic hybrid tubers, suggesting a particular role of this gene in the maceration of resistant tubers.

Individual expression of the pelA, pelE *or* pem *fusions on resistant and susceptible tubers.* The individual expression of the *pelA* and *pelE* fusions was analyzed by measuring the β-glucuronidase (GUS) specific activity after infection of the two lines of tubers in three independent experiments. The evolution of the fusion expression was followed over three days (Table 5). From comparison with the Katahdin tubers, we observed: (i) a very poor expression of the *pelA::gus* fusion in the somatic hybrid tubers during the three days; (ii) a decrease of the *pelE::gus* expression on the somatic hybrid tubers after the first day. The *pelE* fusion expression on the susceptible cultivar appeared to be significantly higher the first day following the inoculation. The levels of expression of these two *pel* fusions are consistent with the decrease of the PL specific activity, resulting from the activity of the five isoenzymes observed in the wild-type *E. chrysanthemi* on resistant tubers (Table 3A).

The *pem::gus* expression was also analyzed in the tissue of susceptible and resistant tubers. After twenty four hours, the *pem::gus* expression was similar in both Katahdin and somatic hybrid tubers, but the second day it increased slightly in the resistant tubers (Table 5). However, the difference was not statistically significant.

Table 5. Expression of the *pel::gus* and *pem::gus* fusions in resistant and susceptible tubers. GUS specific activity was estimated after inoculation with the various fusion strains and is expressed as enzyme units per mg of bacterial dry weight (BDW). The mean value and the standard deviation of 3 independent experiments are given. For the *pelA* mutant carrying the *pelA::gus* fusion, the two factor variance analysis performed indicates that the GUS specific activity is significantly higher on the susceptible potato line (p = 0.0001). For the *pelE* mutant, the data obtained the second and third day are obviously very similar for both cultivars. The data collected from the first day were submitted to the Student test, and the GUS activity was found to be significantly higher on susceptible tubers (p = 0.0015). For the *pem* mutant, the two factor variance analysis performed indicates that the GUS specific activity is not significantly higher on the susceptible potato line (p = 0.14)

Fusion	Time	Katahdin GUS specific activity (unit/mg BDW)	Somatic hybrid GUS specific activity (unit/mg BDW)
pelA::gus fusion	24 h	94.0 ± 10.0	3.8 ± 1.2
	48 h	41.0 ± 6.0	2.8 ± 0.8
	72 h	22.0 ± 9.0	3.8 ± 2.2
pelE::gus fusion	24 h	2605 ± 432	524 ± 162
	48 h	829 ± 227	1110 ± 242
	72 h	142 ± 50	175 ± 92
pem::gus fusion	24 h	127 ± 37	98 ± 35
	48 h	29 ± 2	141 ± 79
	72 h	16 ± 9	26 ± 10

Discussion

In this study we compared two lines of potato tubers with different degrees of resistance to soft-rot caused by *E. chrysanthemi*. We chose the commercial cultivar Katahdin as a very sensitive plant and the somatic hybrid 946, resulting from protoplast fusion between *S. brevidens* and *S. tuberosum*, as a plant with a high level of resistance (Austin et al., 1988, Lojkowska and Kelman, 1994). Our results confirm the partial resistance of somatic hybrid tuber tissue to maceration caused by *Erwinia*. Moreover, comparison of these two lines leads to the conclusion that after infection by *E. chrysanthemi*, the PL activity is lower in the resistant tubers than in the sensitive tubers.

The lower PL specific activity observed in the somatic hybrid tubers may result from a limitation either at the level of gene expression or at the level of the enzyme activity. With *pelA* and *pelE* fusions, we observed a reduced expression in resistant somatic hybrid tubers compared with susceptible Katahdin tubers (Table 5), indicating an important variation at the level of the transcription of these genes. It is clear that these pectinase genes of *E. chrysanthemi* appear to be differentially regulated in the two hosts. Our data suggest that the mechanism of resistance of the somatic hybrid tubers could be linked to the limitation of the PLs production at the transcriptional level. The mechanism of this limitation could be described either as a very efficient induction of the *pel* genes in the susceptible tubers, or as a specific inhibition of the *pel* genes expression in the resistant tubers. The molecular signal(s) responsible for the variation of *pel* gene expression in these two kinds of tubers has yet to be identified. Yang et al. (1992) showed that the induction of the pectic enzymes was reduced under soft-rot incompatible conditions (low moisture), compared with rot compatible conditions (high moisture). This indicates that a poor induction of PL production could result from a particular environmental condition. In our experiments, we controlled two important factors for soft-rot (temperature and humidity). Therefore, the different levels of expression of the *pel* genes result from another factor(s) such as the chemical composition or the microenvironment provided by the two kinds of tubers. In addition to the transcriptional control of the *pel* genes, we can not rule out the possible existence of a PL enzyme inhibitor(s) or activator(s) in the tubers. Wattad et al. (1994) showed that the avocado cultivar resistance is related to the amount of epicatechin, an inhibitor of the *Colletotrichum gloeosporioides* PL enzyme. However, McMillan et al. (1994a) could only isolate an activator, and not an inhibitor, of the *Erwinia carotovora* PLs from potato tubers.

The two *pel* genes, *pelA* and *pelE*, have different patterns of expression on the resistant somatic hybrid tubers. The *pelA::gus* expression is barely detectable throughout the experiment whereas the *pelE::gus* expression is only temporarily affected. These pectinase genes appear differentially regulated in the same host. The possibility for each isoenzyme gene to exhibit a specific regulation is consistent with the existence of five independent transcriptional units for the five *pel* genes. The 3 genes studied, *pelA*, *pelE* and *pem*, are all negatively regulated by the KdgR repressor (Nasser et al., 1994). Since the corresponding fusions are not affected in the same way in the two potato lines, complex regulations beyond the KdgR repression could occur in sensitive or resistant tubers. The expression of each pectinase gene responds to different regulatory circuits that have been partially identified: *pecS*, *pecY*, *pecT* (Reverchon et al., 1990, 1994; Surgey et al., 1996). Various plant extracts are

known to induce the different *pel* genes with variable efficiency: carrot roots, *Diffenbachia* or the fruit of pumpkin (Bourson et al., 1993), potato tubers or plantlets (Lojkowska et al., 1993), *Saintpaulia ionantha* and pea or chicory leaves (Beaulieu et al., 1993; C. Masclaux et al., 1996). The molecular signal(s) responsible for the variation of *pel* gene induction in these different tissues has yet to be identified.

The case of the *pelA* gene is of particular interest. The *pelA* gene was very poorly expressed in somatic hybrid tubers but highly expressed in Katahdin tubers (Table 5) and in another susceptible cultivar, Mona Lisa (Lojkowska et al., 1993). Despite its poor maceration activity on potato tubers, *pelA* is essential for full pathogenicity of *E. chrysanthemi* 3937 on *Saintpaulia* plants, on pea or chicory (Boccara et al., 1988; Beaulieu et al., 1993). The preference of PLa to degrade polymers over oligomers could explain its important role during infection (Preston et al., 1992). Our results suggest that the two susceptible tubers are specifically able to induce the expression of the *pelA* gene, which is barely detectable in both cultures in synthetic media and in resistant potato tubers. The pattern of expression of the *pelA::gus* fusion suggests that PLa plays a special role in the virulence since *pelA* level of expression appears related to the disease severity in tubers.

We observed a significant reduction of the rot extent in the somatic hybrid tubers inoculated with the mutant devoid of PME, compared with the wild-type. Moreover, such a striking reduction of the rot extent was not observed on the susceptible cultivar (Table 4). This result indicates the key role of the *pem* gene in the *E. chrysanthemi* virulence towards resistant tubers. McMillan et al. (1993b) demonstrated that the cell walls of tubers of progeny of the somatic hybrids between *S. brevidens* and *S. tuberosum* have a pectin with a high degree of methylation. Such a pectin, without the action of the PME, could be less accessible to *E. chrysanthemi* pectate-lyases than the weakly methoxylated polymer present in susceptible tubers. As a result the hybrid tubers would limit, extremely efficiently, the maceration caused by a *pem* mutant.

In conclusion, comparison of the expression of the pectinase genes of *E. chrysanthemi* 3937 in susceptible or resistant potato tubers shows that the resistance is related to limitation of PL biosynthesis. Moreover, the relative expression of these genes appears modulated according to the plant tissue. Thus, an adaptative regulation of the different pectinase genes probably contributes to the outcome of the interaction beween the plant and the bacteria.

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