

Role of Endoglucanases in *Erwinia chrysanthemi* 3937 Virulence on *Saintpaulia ionantha*

MARTINE BOCCARA,* JEAN-LUC AYMERIC, AND CHRISTEL CAMUS

Laboratoire de Pathologie Végétale, Institut National de la Recherche Agronomique, 75005 Paris, France

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The role of endoglucanases (endoglucanases Z and Y) in *Erwinia chrysanthemi* pathogenicity on *Saintpaulia ionantha* was assessed by mutagenizing cloned *cel* genes (*celZ* and *celY*) and recombining them with the chromosomal alleles. Strains with an Ω interposon in *celZ*, a deletion in *celY*, or a double *cel* mutant were as virulent as the wild-type strain. However, in the strain with a deletion in *celY*, a delay in the appearance of symptoms was observed, and then maceration progressed as in plants infected with the wild-type strain, suggesting that *E. chrysanthemi* endoglucanases play a minor role in soft rot disease development.

Erwinia chrysanthemi 3937, a soft rot disease plant bacterium, produces and secretes several depolymerizing enzymes, including pectinases, endoglucanases (EGs), and proteases. The major pectate lyases of *E. chrysanthemi* 3937 and pectin methylesterase have been shown to be necessary for full pathogenicity on *Saintpaulia ionantha* (5, 6). However, a mutant of *E. chrysanthemi* 3937 with its five pectate lyase activities deleted has been shown to be noninvasive but was still able to macerate the inoculated leaves of *Saintpaulia* plants and to produce a limited maceration on potato tubers (1, 3), suggesting a putative role for other depolymerizing enzymes produced by *E. chrysanthemi*.

The role of EG in the pathogenicity of *Pseudomonas solanacearum* and *Xanthomonas campestris* has been studied (11, 18). The virulence of an EG-deficient strain depended on the bacterial species. A delay in the appearance of symptoms and killing of tomato plants was observed in an EG-deficient strain of *P. solanacearum*. However, an *X. campestris* mutant lacking EG showed little reduction in virulence.

E. chrysanthemi 3937 produces two EGs: EGZ, which comprises the major activity and is secreted (8), and EGY, which is less abundant and is periplasmic (12). The genes encoding these activities, *celZ* and *celY*, respectively, have been mapped in two different locations of the chromosome, and the conditions of their optimum expression have been studied with *lacZ* fusions (2). To study the contribution of *E. chrysanthemi* EGs in soft rot disease, we chose not to use the previously described *cel* mutants, which were *lacZ* translational fusions (2), but instead constructed new *celY* and *celZ* mutants and analyzed their pathogenicity on *Saintpaulia* plants.

A *celZ* mutant was constructed as follows. The Ω interposon encoding resistance to spectinomycin and streptomycin and carrying transcriptional and translational termination signals was obtained after *Sma*I digestion of the pHP45 Ω plasmid (15). This *Sma*I fragment was inserted into the Klenow fragment-filled *Cla*I site of the *celZ*-carrying plasmid pAJ24 (13) (Fig. 1). The mutated plasmid was introduced into strain 3937 (wild type), and after marker exchange (19), the E-1006 strain was selected.

A *celY* mutant was obtained by following the strategy

described by Ried and Collmer (17). A *Pst*I fragment carrying the *sac* genes of *Bacillus subtilis* and the *npt* gene of *Escherichia coli* was obtained through partial digestion of the pUM24 plasmid and was introduced into the *Pst*I site of the *celY*-containing plasmid pMH17 (7). Exchange recombination between the mutated cloned *celY* gene and the chromosomal allele was then selected. Spontaneous excision of the *npt-sacB-sacR* fragment was then selected in the presence of 1% sucrose. To measure the extent of the deletion, strain E-1008 DNA was hydrolyzed with *Bam*HI, *Sma*I, or *Pst*I. After transfer, the DNA blot was hybridized with the *Bam*HI-*Sma*I fragment from pMH17 (Fig. 1). Strain E-1008, which had lost the *Sma*I site and suffered a deletion of 2 kb overlapping the *celY* gene, was retained (data not shown).

The E-1006 and E-1008 mutant strains were checked by electrofocusing and were found to be negative for EGZ and EGY (Fig. 2), respectively, before their inoculation into *Saintpaulia* plants. To construct a double *celY celZ* mutant (E-1014), the *cel::* Ω mutation carried by the E-1006 strain was introduced into the E-1008 strain by transduction with the phage ϕ EC2 (16). No EG was produced by strain E-1014 (Fig. 2).

When strain 3937 was inoculated into plants, a translucent spot at the inoculation site was visible after 24 h. After 3 days, the maceration had affected all of the leaf blade and sometimes had affected the petiole. A week later, maceration had propagated to the whole leaf (Fig. 3A), and 2 weeks later, the whole plant was diseased (70% systemic responses). When the E-1006 mutant was inoculated on *Saintpaulia* plants, the progression of symptoms of E-1006 infection over a week was identical to that of the wild-type strain. The progression of symptoms was not as rapid when strain E-1008 was inoculated (Fig. 3B). However, a week after inoculation, no difference in the number of diseased plants and the severity of symptoms between the wild type, *cel* mutants, and the double *cel* mutant ($P < 0.05$ [Fig. 4]) was observed.

The mutant E-1006, which is depleted of *E. chrysanthemi* major EG (EGZ), is as virulent as the wild-type strain. In the E-1008 mutant, a slight delay (3 days) in the progression of symptoms was observed, suggesting that EGY is necessary in the early stages of infection. In the infected plant, the bacteria develop in the intercellular space and are in contact with the pectin-rich middle lamella. The delay observed with E-1008 is surprising, because EGY is a minor and periplasmic EG. Recent data on the specificity of cellulolytic enzymes suggest

* Corresponding author. Mailing address: Laboratoire de Pathologie Végétale, INRA, INA P/G, 16 rue Claude Bernard, 75005 Paris, France. Phone: (1) 44 08 17 07. Fax: (1) 44 08 17 00.

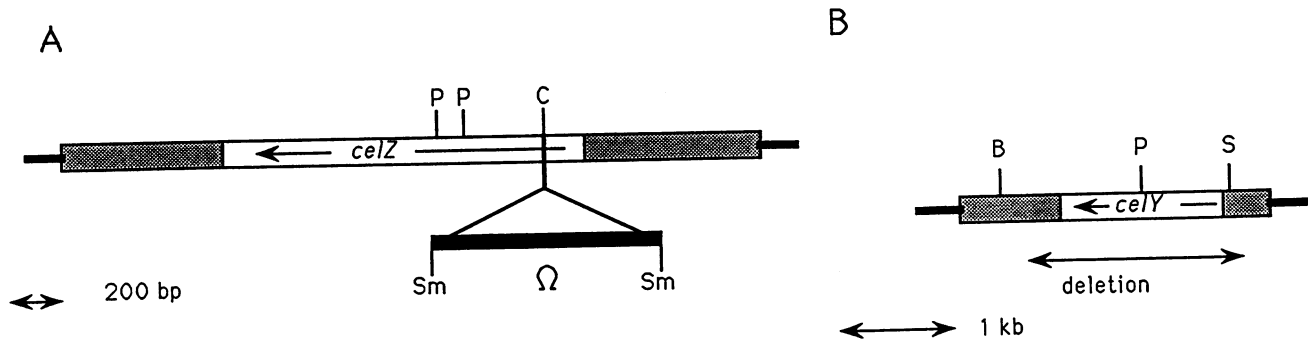


FIG. 1. Physical map of pAJ24 (A) and pMH17 (B) and location of mutation constructed in *celZ* and *celY* genes. The arrow within the genes indicates the direction of transcription. Shaded areas, *E. chrysanthemi* DNA; solid bars, vector DNA. Restriction sites: B, *Bam*HI; C, *Cl*aI; P, *P*sI; Sm, *Sma*I.

that the substrate specificity of EGY is broader than that of EGZ (2a, 9). This could suggest that EGY hydrolyzes other substrates present in the middle lamella, making the pectin more accessible to pectinases. However, as the level of pectate lyase increases (14), EGY is no longer required, and the maceration progresses as in the wild-type strain. Our results suggest that the EGs of *E. chrysanthemi* 3937 are not essential for soft rot disease development on *Saintpaulia* plants. Thus, the role of the EG might be limited to the saprophytic life of *E. chrysanthemi*; on dead tissues cellulose already attacked by fungi might be a better substrate for bacterial EGs, providing nutrients for the bacteria.

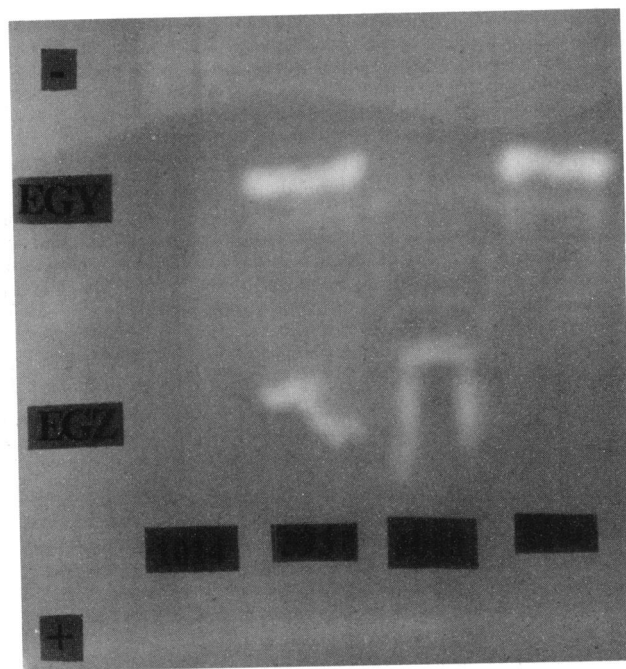


FIG. 2. Electrofocusing on a thin polyacrylamide gel of each of the *cel* mutants (E-1006 [*celZ*], E-1008 [*celY*], and E-1014 [double *cel* mutant]) and wild-type strain 3937. The different enzymes are listed on the side. EGs in the whole-cell extract of stationary-phase cells were analyzed (10). Fifteen microliters of each fraction was layered on a thin polyacrylamide gel, and electrofocusing was performed in a pH gradient from pH 3 to 10. EG activities were assessed according to the method of Bertheau et al. (4).

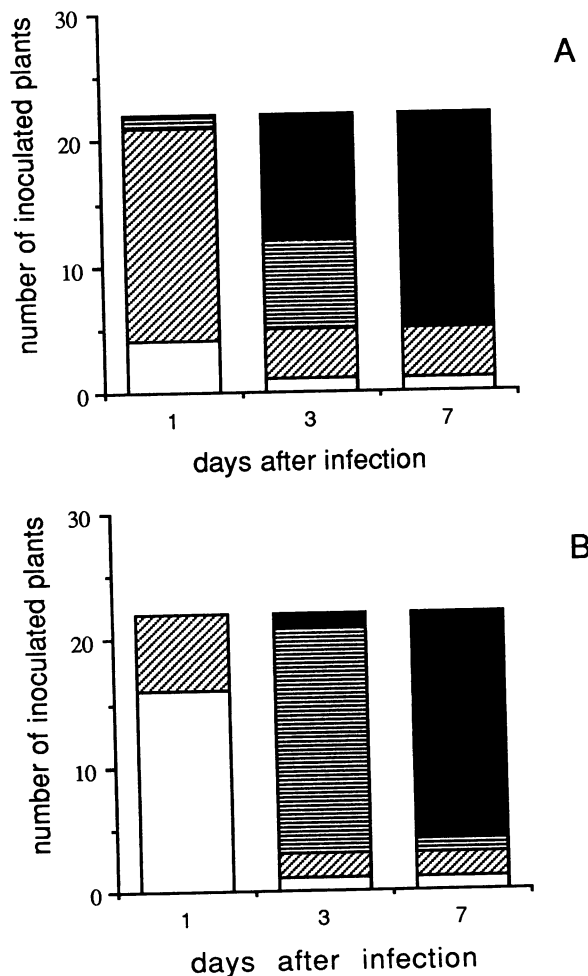


FIG. 3. Progression of symptoms during the first week after inoculation of *Saintpaulia* plants with wild-type strain 3937 (A) or with the E-1008 *celY* mutant (B). ■, leaf blade and petiole macerated; ▨, leaf blade macerated; ▩, maceration in the infiltrated zone; □, no symptoms. Three-month-old *Saintpaulia* plants were inoculated by infiltrating, with a needle, the leaf parenchyma with about 2×10^7 bacteria ($100 \mu\text{l}$). The plants were cultivated at 29°C in a growth chamber with high humidity and a light intensity of 6,500 lx with a photoperiod of 16 h of light and 8 h of dark. The results of one experiment are presented (three repetitions).

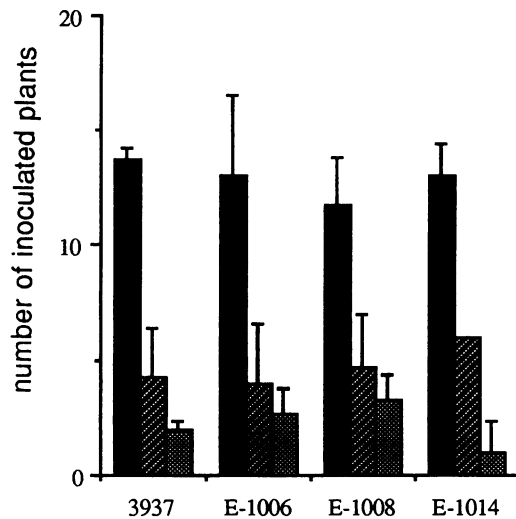


FIG. 4. Distribution of responses induced by wild-type and mutant strains on *Saintpaulia* plants. Symptoms produced after 1 month were recorded. The symptoms were classified into three groups: ■, systemic response (the whole plant is macerated); ▨, localized response (maceration limited to the inoculated leaf); and □, local necrosis or no symptoms. The results are averages of three to five experiments; each experiment was performed with 20 plants. Standard error bars are indicated. Analysis of variance demonstrated that there were no significant differences between the means of the three groups ($P < 0.05$).

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