Self-regulation of Pir, a Regulatory Protein Responsible for Hyperinduction of Pectate Lyase in *Erwinia chrysanthemi* EC16

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Previously, we have cloned and characterized the *pir* (plant inducible regulator) gene, which is responsible for hyperinduction of the synthesis of an isozyme of pectate lyase (PL) in *Erwinia chrysanthemi* EC16 in the presence of potato extract and sodium polypectate (NaPP). The Pir protein purified from *Escherichia coli* overexpressing *pir* is able to bind to the promoter region of *pir* as a dimer. Self-regulation of *pir* by its own translational product (Pir) was suggested from the findings that Pir binds at the promoter region of *pir* and that the hyperinduction of the *pir-lux* construct in response to plant extract was observed only in *pir*⁺ but not in *pir* mutant EC16. Thus, hyperinduction of PL was thought to be mainly due to overproduction of Pir. On the other hand, KdgR and PecS, which have been reported to be the major regulatory proteins for the synthesis of pectic enzymes, did not bind to the promoter region of *pir*. Thus, the regulation of Pir synthesis seems to be independent of KdgR and PecS. Also, its expression was insensitive to catabolite repression as predicted from failure of cyclic AMP (cAMP)-CRP (cAMP recognizing protein) to bind at the *pir* promoter region.

Erwinia chrysanthemi is a phytopathogenic bacterium that causes soft-rot disease in a wide variety of host plants (Pérombelon and Kelman 1980). Its pathogenesis depends on production and secretion of depolymerizing enzymes such as pectinase, cellulase, and protease (Barras et al. 1994), and among these cell-wall-degrading enzymes, pectate lyases (PLs) that cleave sodium polypectate (NaPP) in a transeliminative manner have been considered to be the most important (Collmer and Keen 1986). Strain EC16 of *E. chrysanthemi* produces and secretes four major PL isozymes, named PL_A, PL_B, PL_C, and PL_E (Barras et al. 1987; Collmer and Keen 1986). These isozymes are produced from independent genes (*pelA*, *pelB*, *pelC*, and *pelE*; Brooks et al. 1990; Tamaki et al. 1988), and their expression is elevated by growth in the presence of NaPP, which is metabolized to produce the true intracellular inducer, 2-keto-3-deoxygluconate (KDG; Hugovieux-Cotte-Pattat and Robert-Baudouy 1987). This induction mechanism (product induction; Tsuyumu 1977) is mediated by

releasing a repressor, KdgR, from the KdgR-box in the promoter regions of these genes by allosteric change of KdgR after binding with KDG, one of the metabolic products produced from pectic substance.

Recently, we have found a new regulatory protein, Pir, that is specifically responsible for hyperinduction of *pelE* expression in the medium containing NaPP and potato extract (Nomura et al. 1998). In this paper, we show that the purified Pir protein binds at the promoter region of *pir*. From the analysis of induction pattern with the *pir*-reporter gene construct in *pir*⁺ and *pir* mutant backgrounds, the expression of *pir* was shown to be regulated by Pir (self-regulation).

RESULTS

Characterization of the *pir* promoter region.

The transcriptional start site for *pir* was determined to be at G located 52 nucleotides (nt) upstream from the translational start site of *pir* and was the same when EC16 cells were grown either in the presence or in the absence of potato extract (Figs. 1 and 2). Before this transcriptional start site, a σ_{70} type promoter –35 region (TTGCAT, from –36 to –31 nt) and –10 region (CATGAT, from –13 to –8 nt) can be seen (Fig. 1). Thus, the expression of *pir* seems not to be dependent on a new σ factor. The intensity of the band at the transcriptional start was highest when RNA extracted from the cells grown

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-201      DnaI
GGAGAACTTTAAACGGGCGTTGGATGAGCACCCTATCGGTATTCCTTACCCGC
AGATGGATGTGCATCTGCACCATGCTTGTGCCAACCGGATGGGGTCACAAGAA
ACGGCGACGCTGTAAACGTTCCAGCCGGTATCGGCAACAGGACCTAGGCCGTTT
CCTCTATTGTCATCGCATAACATGCTGTTTCATGATGCTCTCGTCCCGCAGAG
          -35          -10          +1
GTTGATGTCTGACGGGCGTAATGAGAGGAAGCTCAGACGTGATAAATGAACA
          SD          M I N E H
TAGCATTGATGACGAGGTGAAAGCGGAAACCGTTCGGGAAGTCAAAGCCTGT
          S I D D E V K A E K P L G S Q S L
                                     +118
  
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Fig. 1. Nucleotide sequence of the promoter region of the *pir* gene of *Erwinia chrysanthemi* EC16. Numbering based on transcriptional start site determined by primer extension (Fig. 2). Translational start site is at +52 nucleotides. The DnaI-AluI fragment (235 bp) is used for the binding assay and construction of *lux* fusion. –35 and –10 regions of σ_{70} type promoter are underlined. Boxed sequence indicates region protected by Pir from DNase I digestion.

under hyperinducing condition (i.e., in the presence of potato extract and NaPP) was used (Fig. 2). This accumulation of Pir should lead to hyperinduction of PLe under the same conditions (Nomura et al. 1998).

DNA-binding assays on the promoter region of *pir* (the 267-bp *XmaI-SphI* fragment; Table 1) with KdgR (Nasser et al. 1991, 1992, and 1994; Reverchon et al. 1991), PecS (Praillet et al. 1996, 1997; Reverchon et al. 1994), and cyclic AMP (cAMP)-CRP (cAMP recognizing protein; Nasser et al. 1997; Reverchon et al. 1997) of *E. chrysanthemi* showed no

Fig. 2. Determination of transcriptional starting point of *pir* by primer extension analysis. Lanes A,C,G,T correspond to the DNA sequence ladders with PIRO-2 as the primer. PIRO-2 was complementary to the sequence from +117 to +97 nucleotides (nt) of the *pir* coding region. Results shown are after primer extension from end-labeled PIRO-2 with total RNA (10 µg) preparations from EC16 cells grown in (lane 1) minimal salts + glycerol, (lane 2) minimal salts + glycerol + NaPP, or (lane 3) minimal salts + glycerol + sodium polyelectate + potato extract.

shifted band (data not shown). Thus, *pir* expression may not be regulated by these regulatory molecules.

Pir binds to the promoter region of *pir*.

When the 267-bp *XmaI-SphI* fragment containing the *pir* promoter region was used as the target DNA in DNA-binding assays in the presence of purified Pir, a single retarded band was observed when Pir was added at a concentration between 30 and 120 nM (Fig. 3). After a 100-fold molar excess of unlabeled *XmaI-SphI* *pir* fragment was added to the binding mixture, Pir-*pir* complex disappeared. The molecular mass of the shifted band in native PAGE (polyacrylamide gel electrophoresis) was calculated to be approximately 60 kDa by the method of Bading (Bading 1988). The region from -38 to -7

Fig. 3. DNA-binding assay on the promoter region of *pir* with purified Pir. The *XmaI-SphI* fragment of *pir* (267 bp) was used as probe for DNA-binding assay. Labeled *XmaI-SphI* fragment (30 fmol) (free, lane P) was mixed with (lane 1) 30, (lane 2) 50, (lane 3) 70, or (lane 4) 120 nM concentrations of purified Pir. Lane 5, same condition as lane 4, except that it contains a 100-fold molar excess of unlabeled *XmaI-SphI* fragment of *pir*.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
Strain		
<i>Escherichia coli</i>		
MV1184	<i>araΔ(lac-proAB) rpsL thi (φ80lacZΔM15), Δ(srl-recA) 306::Tn10(Tet^r), F'[traD36 proAB⁺ lacF⁺ lacZΔM15]</i>	Nippon Gene, Tokyo
BL21 (DE3)	F ⁺ <i>ompT hsdSB (rB⁻ mB⁻) gal dcm</i> (DE3)	Novagen, Madison, WI
<i>Erwinia chrysanthemi</i>		
EC16	Wild type	A. Chatterjee, University of Missouri
K2367	<i>gus-km</i> cassette insertion mutant of <i>pir</i> , Pir ⁻ , Km ^r	Nomura et al. 1998
EP16	EC16 integrants of Tn7- <i>lux pir</i> promoter construct, St ^r	This study
KPIR2367	K2367 integrants of Tn7- <i>lux pir</i> promoter construct, Pir ⁻ , St ^r , Km ^r	This study
Plasmid		
pUC118	Cloning vector, Ap ^r	Nippon Gene
pHSK728	Tn7- <i>lux</i> transcriptional fusion vector, Ap ^r , St ^r	Shen et al. 1992
pMON7181	Helper plasmid carrying genes for Tn7 transposition, Gm ^r	Barry 1988
pET21a-d(+)	T7 overexpression vector, Ap ^r	Novagen
pIEC-1	1.45-kbp <i>EcoRV-KpnI</i> fragment containing <i>pir</i> cloned into the <i>SmaI-KpnI</i> site of pUC118	Nomura et al. 1998
pPIR-PR	235-bp <i>DraI-AluI</i> fragment from pIEC-1 cloned into the <i>SmaI</i> site of pUC118	This study
pPIR-LUX	235-bp <i>DraI-AluI</i> fragment from pIEC-1 cloned into the <i>SmaI</i> site of pHSK728	This study
pET-PIR	1.1-kbp <i>NdeI-EcoRI</i> fragment containing <i>pir</i> open reading frame cloned into the <i>NdeI-EcoRI</i> site of pET21a-d(+)	This study

nt in the *pir* promoter was shown to be protected by Pir from digestion with DNase I in a footprinting experiment (Fig. 4). This sequence (ATAACATGCTGTTTCAT, from -27 to -11 nt) was similar to the sequence (ACAAAATGGCATTTCAT) in the promoter region of *pele* protected from the digestion (Nomura et al. 1998). These results indicated that Pir specifically binds to the region from -38 to -7 nt in the *pir* promoter as a dimer.

Self-regulation of *pir*.

Expression of the *pir-lux* construct in EC16 increased in the presence of either NaPP or potato extract, compared with that in minimal salts + glycerol, but it increased further in the presence of both NaPP and potato extract (Fig. 5). In K2367 (*pir* mutant), however, induction and hyperinduction observed in EC16 were significantly reduced. Since Northern (RNA) blot experiments with a *pir* clone as the probe showed that the transcription of *pir* is monocistronic (data not shown), the *pir* phenotype was thought to reflect the loss of only *pir*. Pir binds to its *pir* promoter region (Figs. 3 and 4) and *pir* expression is reduced in the *pir* mutant. These results suggest that the expression of *pir* was self-regulated in a positive manner by its own translational product only in the presence of potato extract.

In the medium containing NaPP alone, *pir* expression in EC16 and in K2367 was approximately the same. Thus, the induction of the synthesis of Pir in this condition seems to be controlled by a regulatory protein other than Pir. Failure of binding of KdgR and PecS to the *pir* promoter indicates that this regulatory protein should be other than KdgR and PecS. Also, since *pir-lux* expression was high even in the presence of glucose (data not shown), the expression of *pir* is insensitive to catabolite repression. This result is consistent with the absence of binding of CRP at the *pir* promoter region.

Fig. 4. Determination of binding site for Pir on the promoter region of *pir*. DNase I footprinting was performed after binding with purified Pir protein. Labeled *Xma*I-*Sph*I fragment (30 fmol) (free, lane P) was mixed with (lane 1) 80 or (lane 2) 120 nM concentrations of purified Pir.

Plant sources for hyperinduction of Pir.

In EC16, hyperinduction of *pir* was observed when cells were grown in the presence of extracts of not only potato but also celery, Chinese cabbage, and carrot, and hyperinduction was not observed in K2367 (Table 2). Thus, the potential inducing factor(s) for hyperinduction of *pir* that lead to the hyperinduction of PL synthesis may be widely distributed in the plant.

A EPIR16 (wild-type)

B KPIR2367 (*pir*⁻)

Fig. 5. Positive autoregulation of *pir*. Light production by EC16 (A, EPIR16) and *pir* deficient mutant K2367 (B, KPIR2367) carrying the *pir-lux* construct were measured with a chemiluminescence detector. Specific activity of *pir-lux* was expressed as cpm per ml per cell density (cpm/ml/OD₆₀₀). Background value of this detector was about 10³ cpm/ml/OD₆₀₀. Symbols: open square, minimal salts + glycerol; open diamond, minimal salts + glycerol + sodium polypectate (NaPP); open circle, minimal salts + glycerol + potato extract; open triangle, minimal salts + glycerol + NaPP + potato extract. Carbon sources were added to the minimal salts at the following concentrations: glycerol, 2 g/liter; NaPP, 2 g/liter; potato extract, 1%. All cultures were grown on a shaker at 27°C. Each value is the average of at least five determinations.

DISCUSSION

A single start site for the transcription of *pir* was found at 52 nt upstream from the translational start site, when EC16 cells were grown either in the presence or in the absence of potato extract (Fig. 1). Before this transcriptional start site, a typical σ_{70} type promoter was observed. Thus, unlike the case with the *hrp* operon (Fenselau and Bonas 1995; Wengelnik and Bonas 1996), the induction of *pir* expression seems to require no new σ factor in responding to the plant extract. From the hyperinducible nature of Pir synthesis in the medium containing plant extract in addition to NaPP and glycerol, known regulatory proteins for enzymes such as KdgR and PecS were thought to be involved in its regulation. However, since none of these molecules bound to the promoter region of *pir* (data not shown), they seem not to be involved in regulation of Pir synthesis. Also, though many genes including *pel* have been reported to be regulated by catabolite repression with the cAMP-CRP complex as a positive factor (Nasser et al. 1997; Reverchon et al. 1997; Tsuyumu 1979), cAMP-CRP did not bind to the regulatory region of *pir* and the expression of *pir* was insensitive to catabolite repression (data not shown). Thus, the regulation of *pir* expression is distinguishable from the control of expression of the genes encoding pectin catabolizing enzymes.

Addition of extracts of different plants such as celery, Chinese cabbage, or carrot together with NaPP could also enhance the expression of *pir* (Table 2), which led to hyperinduction of pectin catabolizing enzymes production. In the *E. chrysanthemi* strain 3937, partial purification of the plant component that hyperinduces the production of PL has been reported (Bourson et al. 1993). This plant component is thermoresistant, of low molecular mass, and very hydrophilic. The plant component responsible for hyperinduction of Pir synthesis in our study was also thermoresistant and hydrophilic (data not shown). Thus, the hyperinducing factor in plants for *pir* expression may be similar in chemical nature.

DNase I footprinting analysis showed that Pir protects -38 to -7 nt in the *pir* promoter region from DNase I digestion (Figs. 1 and 4). The Pir binding site in the promoter region of *pir* overlapped with the RNA polymerase binding site (-48 to +5 nt; Collado-Vides et al. 1991). Thus, binding of Pir to the

pir promoter was thought to interact with RNA polymerase. Since Pir production was elevated in the presence of plant extract only in the wild type but not in the *pir* mutant (Fig. 5), Pir was thought to act positively on the transcription of *pir* rather than competitively. Pir may also act as an antirepressor, as previously suggested in the case of *pelE* regulation (Nomura et al. 1998). Thus, a high concentration of Pir in the cell inhibits the binding of an unidentified, specific *pir* repressor, which results in enhancement of *pir* gene expression. Future studies of this system will focus on understanding the precise mechanism (direct or indirect) by which Pir activates its own gene expression as well as expression of the *pel* genes.

MATERIALS AND METHODS

Bacterial strains and growth media.

The bacterial strains and plasmids used in this study are listed in Table 1. Strains of *E. chrysanthemi* and of *Escherichia coli* were grown at 27°C in YP medium (1% polypeptone, 0.5% yeast extract, pH 6.8) and at 37°C in LB (Luria-Bertani) medium (1% polypeptone, 0.5% yeast extract, 1% NaCl, pH 7.0), respectively. M63 medium (Miller 1972) supplemented with a carbon source (0.2%) was used as minimal medium for both bacterial genera. Antibiotics were added at the following concentrations; ampicillin (Ap, 100 µg/ml), kanamycin (Km, 150 µg/ml), streptomycin (St, 25 µg/ml), and gentamycin (Gm, 15 µg/ml). A crude plant extract was prepared by centrifugation at 10,000 × g for 10 min of homogenized extracts of plant tissues by grater followed by filtration through 0.45-µm nitrocellulose filters (Kurabo, Osaka, Japan). One hundredth volume of this extract was added to minimal medium.

Primer extension analysis.

Primer extension analysis was performed as described by Ausubel et al. (1987). The oligonucleotide PIRO-2 (5'-dCAGGCTTTGACTTCCCAACGG-3'), which was complementary to the sequence from +117 to +97 nt in the *pir* coding region (Fig. 1), was used as the primer. PIRO-2 was end-labeled with T4 polynucleotide kinase with 100 µCi of [γ -³²P]ATP (3,000 Ci/mmol). Total RNA was isolated with the ISOGEN RNA isolation kit (Wako, Osaka, Japan) from cells grown to late log phase. Ten micrograms of total RNA was annealed with 1 × 10⁶ cpm of the labeled PIRO-2 at 65°C for 2 h in hybridization buffer (10 mM Tris-HCl pH 8.3, 1 mM EDTA pH 8.0, 150 mM KCl). Extension reaction was performed with avian myeloblastosis virus reverse transcriptase (Wako, Osaka, Japan) in the reaction mixture (50 mM Tris-HCl pH 8.3, 25 mM MgCl₂, 50 mM KCl, 3 µg of actinomycin D per ml, 0.2 mM dNTP mix, 5 mM DTT [dithiothreitol]) at 45°C for 1 h. Reaction was stopped by adding 3× volume (150 µl) of stop solution (100 µg of salmon sperm DNA per ml, 20 µg of RNase A per ml). Extended fragments were ethanol precipitated after phenol/chloroform extraction, resuspended in 3 µl of dye mixture, and loaded onto a 6% polyacrylamide-urea sequencing gel. Manual DNA sequencing was done with the T7 polymerase sequencing kit (Pharmacia Biotech, Uppsala, Sweden) with a clone containing the *pir* gene (pIEC-1; Table 1) as the template DNA and PIRO-2 as the primer, and run in the lanes next to those for primer extension. After electrophoresis, the gel was vacuum dried and exposed to HP film (Amersham, Buckinghamshire, UK).

Table 2. Expression of the *pir* gene in various environmental conditions^a

Carbon source	Lux-specific activity (10 ⁴ cpm/ml/OD ₆₀₀) ^b	
	EPIR16 (wild-type)	KPIR (<i>pir</i> mutant)
Glycerol + sodium polypectate (NaPP)	5.7 ± 2.9	12.1 ± 11.9
Glycerol + NaPP + potato extract ^c	596.3 ± 398.4	15.6 ± 10.6
Glycerol + NaPP + celery extract	315.5 ± 130.5	52.3 ± 49.0
Glycerol + NaPP + carrot extract	384.6 ± 110.8	75.7 ± 28.6
Glycerol + NaPP + Chinese cabbage extract	669.2 ± 353.9	19.8 ± 13.5

^a Light production by EC16 (EPIR16) and *pir*-deficient mutant K2367 (KPIR2367) carrying the *pir-lux* construct was measured by a chemiluminescence detector. All cultures were grown on a shaker at 27°C until optical density at 600 nm of the culture reached at 0.8.

^b Specific activity of *pir-lux* was expressed as cpm per ml per cell density (cpm/ml/OD₆₀₀). Background value of this detector was about 10³ cpm/ml/OD₆₀₀. Each value is average of at least three determinations.

^c Plant extracts (final concentration at 1%) were added into the minimal medium containing glycerol (2 g/liter) and NaPP (2 g/liter).

Purification of Pir protein after overexpression.

With the two oligonucleotides PIRO-3 (5'-AGCTCACATATGATAAATGAACATAGC-3') and PUC-3 (5'-GGAAACA GCTATGACCATG-3') as the primers, a 1.1-kbp DNA fragment containing the *pir* open reading frame with an *NdeI* site at the 5' end and an *EcoRI* site at the 3' end was obtained after polymerase chain reaction (PCR) with pIEC-1 as the template DNA (Table 1). After amplification, an *NdeI-EcoRI* fragment was cloned into the corresponding sites of T7 overexpression vector pET21a-d(+) (Novagen, Madison, WI). After confirmation of absence of mutation, this construct (pET-PIR) was introduced into *E. coli* BL21 (DE3) by electroporation (18.4 kV/cm, 300 μ F, and 4 ohms). Pir was purified from 100 ml of *E. coli* BL21 (DE3) cells grown in LB broth with 150 μ g of Ap per ml. When the optical density at 600 nm of the culture reached 0.8, 0.6 mM IPTG (isopropyl-D-thiogalactopyranoside) was added to the culture to induce the expression of *pir* for 2 h. The Pir protein was purified after ammonium sulfate precipitation (25 to 40%) and HEPARIN POROS column (Roche Diagnostics, Mannheim, Germany) chromatography. Conditions of these purification steps were previously described (Nomura et al. 1998). The concentration of purified Pir was determined by the Bradford kit (Bio-Rad, Hercules, CA) and was stored in a frozen state (-20°C) in buffer A (Nomura et al. 1998) with 50% glycerol. The yield of this two-step purification procedure was approximately 1 mg from 100 ml of culture.

DNA-binding assay and DNase I footprinting.

DNA-binding assays and DNase I footprinting were performed as described previously (Nomura et al. 1998).

Recombinant DNA techniques.

Preparation of plasmid DNA, restriction digestion, ligation, and DNA electrophoresis were done as described by Sambrook et al. (1989). Restriction and modifying enzymes were obtained from Nippon Gene (Toyama, Japan) and New England Biolabs (Beverly, MA).

Insertion of *pir* promoter-*lux* fusion into a chromosome of *E. chrysanthemi*.

A 235-bp *DraI-AluI* fragment containing the *pir* promoter region (-192 to +43 nt; Fig. 1) was ligated into the *SmaI* site of pHSK728 (Shen et al. 1992) to construct the *pir-lux* fusion. The *pir-lux* construct was inserted into the single attachment site on the chromosome of EC16 and *pir*-deficient mutant K2367 by the Tn7 transposition system (Barry 1988; Gold et al. 1992; Nomura et al. 1998).

Bioluminescence assay.

Light production in 10 s by EC16 containing the *pir-lux* construct on its chromosome was measured 3 times every hour by the chemiluminescence detector CLD-100 (Tohoku Electronic, Sendai, Japan). The mean of 3 readings (cpm) in 1 ml was divided by optical density at 600 nm and used as the specific luciferase activity (cpm/ml/OD₆₀₀).

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