

Plant ferredoxin-like protein (PFLP) exhibits an anti-microbial ability against soft-rot pathogen *Erwinia carotovora* subsp. *carotovora* in vitro and in vivo

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Received 23 May 2005; received in revised form 23 December 2005; accepted 3 January 2006

Available online 6 February 2006

Abstract

The anti-microbial protein was frequently used to control the plant bacterial disease. In this study, it was investigated that the anti-microbial activity of recombinant plant ferredoxin-like protein (PFLP) both in vitro and in vivo. The in vitro assay demonstrated that PFLP produced by transformed *Escherichia coli* exhibited an anti-microbial activity against several bacteria strain including *E. coli*, *Erwinia carotovora* and *Pseudomonas syringae*. The effectiveness of this anti-microbial activity was depending on the FeSO₄ that was applied in the cultivated medium. PFLP lost its anti-microbial activity when it was mutated in the 86th cysteine residue that responding for iron binding. Besides, soft-rot symptom of tobacco plants infected by *E. carotovora* was reduced by application of recombinant PFLP. Transgenic tobacco ectopically over expressing PFLP in the cytoplasm protected plant from the infection of *E. carotovora* during the initial stage. These results indicate that PFLP is an anti-microbial protein that might be able to control the plant diseases via reducing the growth of bacterial pathogen.

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Keywords: Plant ferredoxin-like protein (PFLP); Iron competition; *Erwinia carotovora* subsp. *carotovora*; Anti-microbial ability

1. Introduction

Ferredoxin (Fd) functions in fundamental metabolism such as photosynthetic electron transfer proteins, nitrate reduction, and carbon–sulfur metabolism [1]. At least six different Fd isoforms from various green and nongreen tissues have been described so far. They are different in both amino acid sequences and biochemical characteristics from heterotrophic sources [2–6]. Fd I is encoded by an intron-less single-copy gene and exists in the photosynthetic tissues of plant. In recently study, it has been reported that sweet pepper Fd I-like protein (PFLP) was involved in the plant defense mechanism [7]. Transgenic plants over expressing *pflp* gene in tobacco, orchid and rice exhibited a highly disease resistance against to

infection of bacterial pathogen including *Erwinia carotovora*, *Pseudomonas syringa* and *Xanthomonas oryza* [8–10].

Fd I contains several prosthetic iron–sulfur clusters usually. It can be of the [2Fe–2S], [4Fe–4S], or [3Fe–4S] variety and has reduction potentials ranging from –600 to +200 mV [11]. The cluster irons are bridged by inorganic sulfur atoms and are ligated to the polypeptide backbone by the thiolate side chains of cysteine residues [12,13]. For example, the Fe identified from *Anabaena* has four cysteines are present in C41, C46, C49, and C79. In the peptide sequence of PFLP, three cysteine residues exist at 86th, 91th and 94th in the [2Fe–2S] domain respectively [14]. The other domain is casein kinase II (Ck2) phosphorylation site that containing the general consensus sequence S/TXXD/E. It locates in residues from 136 to 139th in carboxyl-terminal region of PFLP [7]. Post-translation regulation of protein by CK2 kinase would help protein to destabilize alpha helices and to counteract caspase cleavage [15,16]. It is uncertain that which domain of iron–sulfur clusters or Ck2 phosphorylation site of PFLP plays an important role in the plant defense.

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Iron competition is an efficient strategy to protect plant from pathogen infection because iron nutrition would influence the microbial community structure in host [17]. For example, the iron-chelator such as lactoferritin [18–22] and siderophore [23–25] was used to control the plant disease. In this study the anti-microbial activity of recombinant PFLP and its mutant were studied *in vitro*. The symptom inhibiting ability of recombinant PFLP was also investigated in the mesophyll space of wild type tobacco leaf. Furthermore, the transgenic tobacco ectopically over expressing PFLP in the cytoplasm was inoculated with *E. carotovora* to investigate the anti-microbial activity of PFLP *in vivo*.

2. Materials and methods

2.1. Plasmid construction

The signal-peptide truncated *pflp* gene (*d-pflp*) was amplified from the genomic DNA of sweet pepper by polymerase chain reaction (PCR) with the following primers: B5-PFLP: 5'-CGG GAT CCC G-AT GGC TTC ATA CAA AGT GAA ACT ACT-3', and S3-PFLP: 5'-CGA GCT CGT T-AG CCC ACG AGT TCT GCT TCT-3'. The *d-pflp*-GC gene was amplified from *d-pflp* by PCR with the following primers: B5-PFLP: 5'-CGG GAT CCC G-AT GGC TTC ATA CAA AGT GAA ACT ACT-3', and S3-PFLP-GC: GAC AGC ACC TGC CCA GGA ATA AGG AAG. The products of primary PCR were used as 5' primers to extending the full-length sequence of *d-pflp*-GC from *d-pflp* by second PCR with the 3' primer S3-PFLP (CGA GCT CGT T-AG CCC ACG AGT TCT GCT TCT-3'). The *d-pflp*-A549 gene was amplified from *d-pflp* by PCR with the following sequence as primers: B5-PFLP: 5'-CGG GAT CCC G-AT GGC TTC ATA CAA AGT GAA ACT ACT-3', and S3-PFLP: CGA GCT CGT T-TC C CTC CTC TAA TTG GTC-3' (Fig. 1a). The amplified fragments of *d-pflp*, *d-pflp*-GC and *d-pflp*-A549 were inserted into the bacterial expression vector pQE30 and transformed into *Escherichia coli* M15.

2.2. Expression of recombinant PFLP protein

E. coli M15 harboring the gene of *d-pflp*, *d-pflp*-GC or *d-pflp*-A549 was incubated in LB medium (Difco, USA) with 100 µg/ml ampicillin and 25 µg/ml kanamycin at 37 °C respectively. When the culture reached an absorbance of 0.6

at A_{600} , isopropylthio-β-D-galactoside induction was given to a final concentration of 1 mM. Cells were harvested after 4 h incubation at 37 °C. Protein was purified with Ni-NTA resin spin kit (Qiagen) in native condition according to the manufacturer instruction. The imidazole residue was removed by P6 gel spin column (Bio-Rad) and the eluting product was dialyzed against 10 mM sodium phosphate buffer (pH 7.0). Purified protein was analyzed in 15% SDS-PAGE.

2.3. Analysis of PFLP anti-microbial activity *in vitro*

Bacterial suspension of *E. coli*, *E. carotovora* subsp. *carotovora* and *Pseudomonas syringae* pv. *syringae* were incubated in the NB medium overnight (8 g/l Nutrient Broth, Difco, USA). Bacterial suspension of them was diluted with sterilized water to 1×10^4 cfu/ml and incubated in the 1/4NB medium (2 g/l Nutrient Broth, Difco, USA) containing different concentrations of purified PFLP (0.06, 0.12, 0.24 and 0.48 µM) at 28 °C with shaking at 175 rpm for 36 h.

Bacterial suspension of *E. carotovora* subsp. *carotovora* (1×10^4 cfu/ml) was incubated in the 1/4NB medium containing 0.2 µM of PFLP, PFLP-GC and PFLP-A549. The multiplication of bacteria was recorded by spectrophotometer O.D.₆₀₀ at following time. PFLP pre-treated with boiling water for 15 min, bovine serum albumin (BSA) and sterilized water were used as the control.

2.4. Analysis of PFLP anti-microbial activity in iron-defective medium

Bacterial suspension of *E. carotovora* subsp. *carotovora* (1×10^4 cfu/ml) was incubated in the XCM minimal medium (20 mM NaCl, 0.16 mM KH₂PO₄, 10 mM (NH₄)₂SO₄, 10 mM fructose, 5 mM MgSO₄, 1 mM CaCl₂, 0.32 mM K₂HPO₄, 10 mM sucrose, 0.03% casamino acid) with or without 0.2 µM d-PFLP at 28 °C, 175 rpm. Different concentrations of FeSO₄ (0, 0.1 and 0.01 mM) were added into this XCM medium. The multiplication of bacteria was recorded by spectrophotometer O.D.₆₀₀.

2.5. Semi-*in vivo* anti-microbial activity analysis of PFLP in tobacco leaf

E. carotovora subsp. *carotovora* were incubated in the NB medium (Difco, USA) overnight. Bacterial suspension of

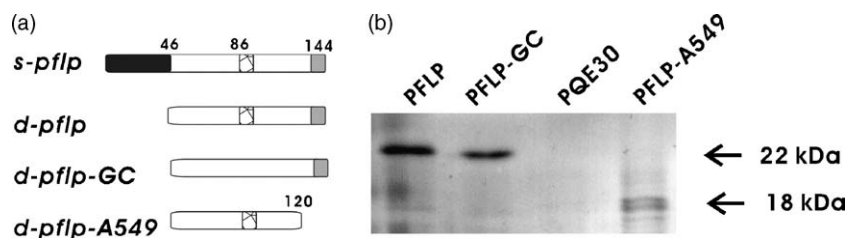


Fig. 1. Recombinant proteins of PFLP, PFLP-GC and PFLP-A549 prepared from transformants of *E. coli* M15. (a) Schematic diagrams of gene constructs. cDNA of *d-pflp*, *d-pflp*-GC and *d-pflp*-A549 were created by PCR and cloned into the pQE30 vector with restriction enzymes *Bam* HI and *Hind* III. (b) Silver staining of purified proteins in SDS-PAGE. Proteins were purified from *E. coli* M15 harboring pQE30 plasmid that containing cDNA of *d-pflp* (PFLP), *d-pflp*-GC (PFLP-GC), *d-pflp*-A549 (PFLP-A549) and vector (pQE30 alone). The purified proteins were separated in 15% (w/v) SDS-PAGE and visualized by silver staining.

carotovora subsp. *carotovora* (1×10^8 cfu/ml) was mixed with different concentrations of purified PFLP (0, 0.25, 0.5, 1.0 and 2.0 μ M) and infiltrated into wild type tobacco leaf, respectively. Photograph was taken 1-day post-inoculation. The soft-rot symptom areas of treated leaves were examined and compared.

2.6. Generation of *d-pflp* transgenic tobacco lines

The PCR product of *d-pflp* gene was digested with *Bam*HI and *Sac*I, and inserted into the PBI121 vector (Clontech, Palo, CA, USA) to replace the *gus* gene. This plasmid was transformed into *Agrobacterium tumefaciens* C58C1 [26]. Transformation of tobacco (*Nicotiana tabacum* cv. *Xanthi*) was performed by the standard leaf disc transformation method using kanamycin selection (100 μ g/ml) [27]. PCR analysis and DNA gel blotting were used to confirm six independent transformant lines. All transgenic plants grew in a the growth chamber (16 h light/8 h dark at 30 °C). The irradiance of growth chamber is 48 μ mol m⁻² s⁻¹. Two independent *d-pflp* transgenic lines were self-fertilized and the seeds were collected.

2.7. Southern blot analysis

Genomic DNA was extracted from young leaf tissue by the genomic kit (Qiagen, Germany). The purified DNA was digested with restriction enzymes *Eco*RI and electrophoretic separation on agarose gels. The Southern blot analysis was performed following the standard procedures [28]. Nylon membranes (Roche, Germany) were hybridized at 65 °C probing with *npf*II gene labeled with digoxigenin-11-dUTP (Roche, Germany). After hybridization, membranes were washed under high stringent conditions (2 \times SSC, 0.1% SDS) and detected by DIG luminescent detection kit (Roche, Germany).

2.8. Western blot analysis of *d-pflp* transgenic tobacco

Total protein of *d-pflp* transgenic tobacco leaf was extracted (0.4 g/ml) in Tris–HCl buffer (150 mM NaCl, 50 mM Tris pH 7.5). The concentration of the sample protein was determined with coomassie brilliant blue dye (BioRad). Protein sample (3 μ g) was subjected to electrophoresis in 15% SDS-polyacrylamide gel (SDS-PAGE). These gels were either stained with coomassie blue or electro-transferred onto nylon membranes (Roche, Germany) with electro-transfer tank (BioRad). Western blots were done with primary antiserum against PFLP.

2.9. Soft-rot pathogen inoculated in the *d-pflp* transgenic tobacco

Four independent leaves of T2 progenies of *d-pflp* transgenic tobacco plants (approximately 30 days old) were infiltrated with 100 μ l bacterial suspension (1×10^5 cfu/ml) of *E. carotovora* subsp. *carotovora* by needle. Photograph was taken 1-day post-inoculation. The infected leaves were bruised and mixed with sterilized water (0.2 cm²/ml). The mixtures were plated and incubated in NB agar plate overnight at 30 °C. The colonies were counted 24 h after incubation.

3. Results

3.1. Construction and expression of PFLP

The *pflp* gene was cloned to the commercial expressing vector pQE30 (Fig. 1a) and transferred into the *E. coli* M15. In the result, the recombinant protein mainly existed in fractions eluted with 250 mM imidazole when it was purified with Ni–NTA his₆ spin column under native condition. In the cloning process, the signal peptide of PFLP in the N-terminal region from 1 to 46th residues was removed artificially because it could not be removed by itself in *E. coli*. The eluent of recombinant PFLP appeared the molecular weight of 22 kDa on the 15% SDS–PAGE that is the same with native PFLP purified from plant. PFLP-GC is a mutant protein from PFLP whose 86th residue was replaced from cysteine to tryptophan. PFLP-A549 is another mutant protein that was truncated at carbonyl-terminal region from residues of 120th to 144th. The eluting product of PFLP-GC appeared in the molecular weight of 22 kDa, but PFLP-A549 could not result in a simple band in SDS–PAGE (Fig. 1b).

3.2. In vitro anti-microbial activity analysis of PFLP

Nutrient broth (NB) medium is good for bacterial growth in vitro but it is too rich in nutrition for anti-microbial activity assay of PFLP. Accordingly, 1/4NB medium that four-fold diluted from NB medium with sterilized water was used for anti-microbial assay of PFLP in vitro. Under this conduction, 0.24 μ M of PFLP inhibited bacterial growth of *E. coli*, *E. carotovora* and *P. syringae* (Fig. 2). The inhibiting activity was increased depending on the dosage of PFLP applied in the 1/4NB medium. Under the same condition, BSA was used as a control that did not show any inhibition activity of bacterial growth.

The anti-microbial activities of two mutated proteins, PFLP-GC and PFLP-A549, were examined as well in vitro. As described before, bacterial growth of *E. carotovora* was inhibited by 0.24 μ M PFLP 12 h after incubation. PFLP-A549

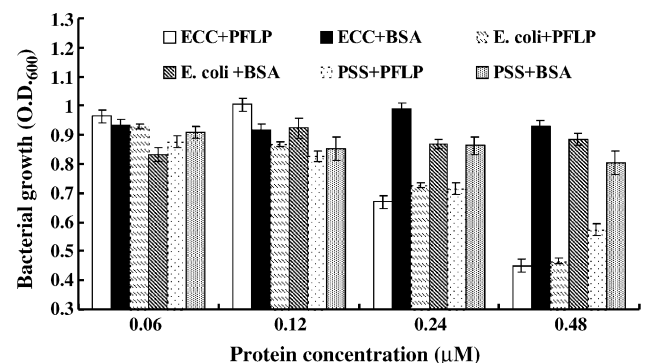


Fig. 2. Anti-microbial activity analysis of PFLP in vitro. Bacterial suspension (1×10^4 cfu/ml) of *E. coli* (*E. coli*), *E. carotovora* subsp. *carotovora* (ECC) and *P. syringae* pv. *syringae* (PSS) were incubated with various concentration of PFLP (0.06, 0.12, 0.24, 0.48 μ M) in the 1/4NB medium at 28 °C. The same concentration of bovine serum albumin (BSA) was used as the control. The bacterial multiplications were measured by spectrophotometer O.D.₆₀₀ 36 h post-inoculation. Vertical error bars denote standard deviation ($n = 4$).

exhibited its anti-microbial activity as good as PFLP under the same conditions. The bacterial growth inhibited by PFLP and PFLP-A549 was standing for 48 h. On the contrary, PFLP-GC could not inhibit bacterial growth in the concentration of 0.24 μM (Fig. 3).

The anti-microbial activity of iron-binding protein is apparent in the iron-defective medium [19–22]. In this study, an iron-defective medium (XCM medium) was used to test the anti-microbial activity of PFLP. Bacterial growth of *E. carotovora* has been inhibited by 0.2 μM of PFLP in the XCM medium until 24 h after incubation. Excess iron exogenously provided by adding 0.01 mM FeSO_4 in the XCM medium has improved the bacterial growth of *E. carotovora*. However, under this condition the improvement of bacterial growth was diminished by the present of 0.2 μM of PFLP. The anti-microbial activity of PFLP was broken if the applied FeSO_4 in XCM medium was raised to the concentration of 0.1 mM (Fig. 4). These results demonstrated that the anti-microbial activity of PFLP was depending on the concentration of free iron in the iron-defective medium.

3.3. Semi-in vivo anti-microbial activity assay of PFLP

E. carotovora is a bacterial pathogen causing a soft-rot symptom in the tobacco leaf. Different concentrations of PFLP were mixed with the bacterial suspension (1×10^8) and infiltrated into tobacco leaf. The area of soft-rot symptom caused by the bacterial mixture was calculated (Fig. 5). The results showed that symptom caused by the mixture of bacteria suspension and 0.5 μM PFLP would be 20% less compared with the absence of PFLP in the bacterial suspension. More than 50% area of the soft-rot symptom was reduced by PFLP when the applied concentration of PFLP in the bacterial suspension was raised to 2 μM .

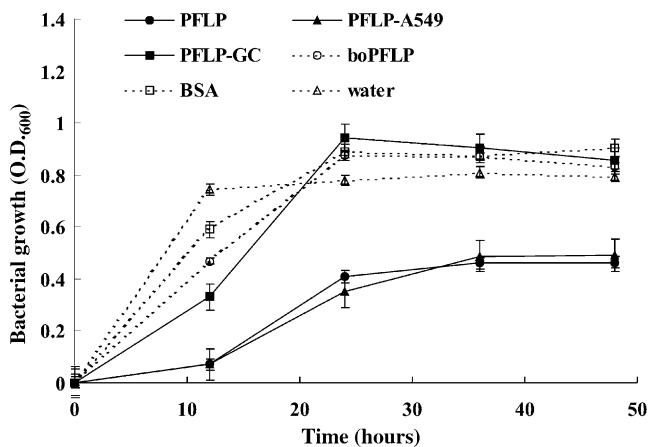


Fig. 3. Anti-microbial activity analysis of mutated PFLP protein in vitro. Bacterial suspensions of *E. carotovora* subsp. *carotovora* (1×10^4 cfu/ml) were incubated with 0.2 μM of PFLP, PFLP-GC or PFLP-A549 respectively in the 1/4NB medium. PFLP pre-treated with boiling water for 15 min (boPFLP), bovine serum albumin (BSA) and sterilized water (water) were used as the controls. The bacterial growth was measured by spectrophotometer O.D.₆₀₀ at a different time point. Vertical bars represent standard deviations ($n = 4$).

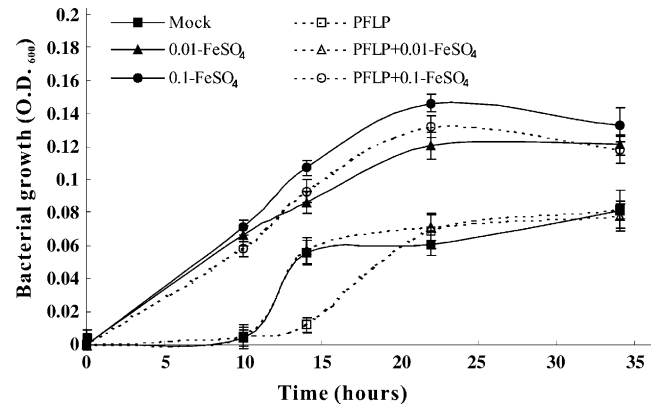


Fig. 4. Anti-microbial activity analysis of PFLP in the iron-defective medium. Bacterial suspension of *E. carotovora* (1×10^4 cfu/ml) was incubated with (PFLP) or without (Mock) 0.2 μM PFLP in the XCM medium containing 0 mM, 0.01 mM (0.01- FeSO_4) or 0.1 mM FeSO_4 (0.1- FeSO_4), respectively. The bacterial concentrations were measured by spectrophotometer O.D.₆₀₀. Vertical bars represent standard deviations ($n = 4$).

3.4. Anti-microbial activity assay of PFLP in vivo

To analyze the anti-microbial activity of PFLP in vivo, the PFLP was over-expressed in the cytoplasm of transgenic tobacco. The independent d-*pflp* transgenic lines were analyzed by Southern blot (Fig. 6a). The results of Southern blot showed that genomic DNA isolated from line T-dPFLP138 exhibited two individual bands in 5.2 and 8.3 kb and only one band in 6.5 kb from line T-dPFLP9-1. These results indicated that the d-*pflp* transgenic lines of T-dPFLP9-1 and T-dPFLP138 were independent lines.

Protein expressing level of PFLP in each transgenic line was compared by Western blot analysis (Fig. 6b). The result showed that protein level of PFLP in transgenic tobacco has 2–3-fold

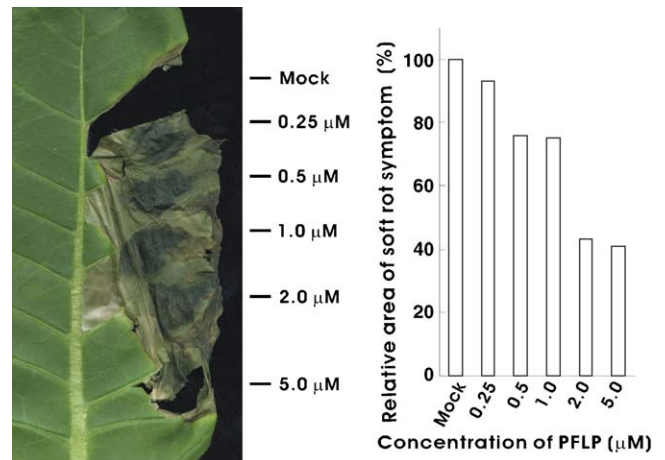


Fig. 5. Anti-microbial activity assay of PFLP in mesophyll space of wild type tobacco. Bacterial suspension of *E. carotovora* subsp. *carotovora* (1×10^8 cfu/ml) was mixed with different concentrations of PFLP (0, 0.25, 0.5, 1.0, 2.0 and 5.0 μM) and infiltrated into mesophyll space of wild type tobacco leaf. Symptoms were photographed and relative area of soft-rot symptom was measured at 1-day post infiltration.

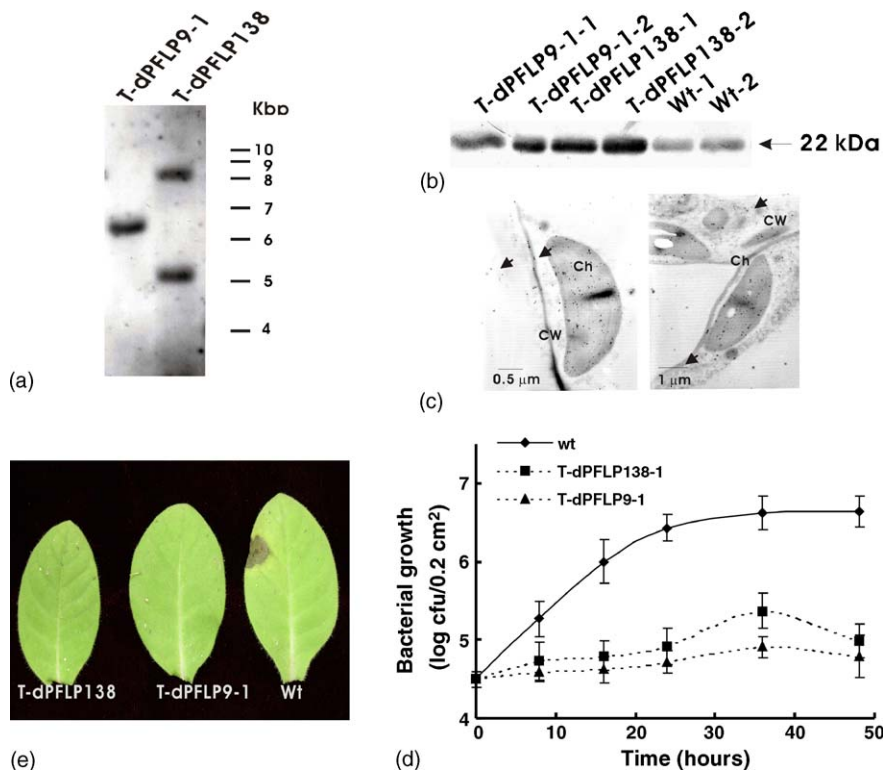


Fig. 6. Soft-rot pathogen inoculated in the *d-pflp* transgenic tobacco. (a) Southern blot analysis of *d-pflp* transgenic tobacco. Genomic DNA (15 μg) of independent *d-pflp* transgenic tobacco lines (T-dPFLP138 and T-dPFLP9-1) were digested with *EcoRI*. Hybridization was performed with the probe against to *npt II* gene at 65 $^{\circ}\text{C}$. (b) Western blot analysis of *d-pflp* transgenic tobacco. Total proteins (3 μg) prepared from wild type and *d-pflp* transgenic tobaccos were analyzed by Western blot probing with antiserum against to PFLP. (c) Electron micrographs of PFLP in *d-pflp* transgenic tobacco. For immunolocalization of PFLP, ultra thin sections of leaves were incubated with antiserum against to PFLP then detected with gold-conjugated antibodies. The cell wall (Cw) and chloroplast (Ch) of tobacco are labeled in the photograph. Arrows indicated the positions of the 12 nm gold particles in the cytoplasm. (d) Soft-rot pathogen inoculated in the *d-pflp* transgenic tobacco. Fully expanded leaves of transgenic (T-dPFLP138, T-dPFLP9-1) and non-transgenic tobacco (Wt) were infiltrated with 100 μl bacterial suspension of *E. carotovora* subsp. *carotovora* (1.0×10^5 cfu/ml). Photograph was taken 1-day post-inoculation. (e) Bacterial populations in the *d-pflp* transgenic tobacco. Bacterial were inoculated as described in (d). The bacterial population was detected at successive time points. Vertical bars represent standard deviation ($n = 4$).

increased compared with wild type tobacco (Table 1). Distribution of over-expressed PFLP in transgenic tobacco was monitored in sub-cellular level. The immunogold-labeled PFLP particles were found both in the cytoplasm and chloroplast of transgenic tobacco (Fig. 6c).

The transgenic tobacco was challenged with soft-rot bacterial pathogen *E. carotovora* (1×10^5 cfu/ml) that caused a macerating symptom in leaf of wild type tobacco. No symptom was observed, however, after bacterial suspension was inoculated in the transgenic tobacco (Fig. 6d). The bacterial population of *E. carotovora* was inhibited to the level of 1×10^5 cfu/ml in the transgenic tobacco, but it was raised to 5×10^6 cfu/ml in the wild type tobacco 48 h after inoculation (Fig. 6e).

Table 1
PFLP levels in *pflp* transgenic tobacco

Transgenic line	Protein level (fold) ^a
T-dPFLP9-1	2.60 \pm 0.41
T-dPFLP138	2.98 \pm 0.36
Wt	1.00 \pm 0.21

^a Protein levels were counted with Western blot. The protein level of PFLP in wild type tobacco was defined as 1.

4. Discussion

In previous study, anti-microbial agents could be used to control the plant disease. For example, thionin that is a toxin protein inhibited enzyme activity of bacterial [29], LTP that is cysteine-rich toxic peptides with low-molecular weight [30] and lactoferrin that is an iron-binding protein competing iron with pathogen from the environment [31]. Here we described a recombinant PFLP that has an anti-microbial activity against growth of *E. coli*, *E. carotovora* and *P. syringa* in vitro (Fig. 2). PFLP also inhibits some other un-described bacteria such as *P. syringae* pv. *tobaci*, *X. campestris* var. *vesicatoria*, and *X. campestris* var. *campestris* (data not shown). These results imply that PFLP is bioactive as a high potential anti-microbial agent such as lactoferrin, thionin and LTP that was used for controlling the bacterial plant disease.

Functional domain of PFLP in the anti-microbial activity was studied. The 86th residue encodes an amino acid of cysteine in [2Fe–2S] domain that responding for iron-binding. PFLP mutated in the 86th residue, designated as PFLP-GC, has lost the anti-microbial activity (Fig. 3). This result implies that the iron-binding capability of PFLP was required for anti-microbial activity against the bacterial growth in vitro. Accordingly, if the

applied concentration of PFLP-GC in cultured medium was increased up to 1 μM , bacterial growth has been inhibited (data not shown). We suppose that PFLP-GC contains some other putative iron-binding residues such as 91th and 94th besides of 86th residues. Thus application of high-concentration PFLP-GC in cultured medium could inhibit bacterial growth. One the contrary, the carboxyl-terminal region of PFLP in mutant PFLP-A549 including the Ck2 phosphorylation site (S/TXXD/E) was truncated. This mutant remains the anti-microbial activity as well as PFLP has. This result excludes the hypothesis that phosphorylation site of PFLP is necessary for anti-microbial activity. Even the Ck2 phosphorylation site of PFLP is not necessary anti-microbial activity, but it might play an important role in protein stability. Therefore the purified protein of PFLP-A549 degrades very soon and could not result a unitary band in the SDS-PAGE (Fig. 1b).

The bacterial pathogen *E. carotovora* uptakes free iron released from the debris of plant by cell wall-degrading enzymes in the initial period of infection [32]. Iron is an essential nutrition for the bacterial growth and pathogenesis of *E. carotovora*. For this reason, iron-binding protein was usually used to inhibit bacterial growth in the iron limiting environment [19–22]. In this study, the PFLP has an iron binding domain that essential for anti-microbial activity. The anti-microbial activity of PFLP is depending on the existing concentration of FeSO_4 in the iron-defective medium (Fig. 4). Mesophyll space is also an iron-limited environment. The recombinant PFLP has inhibited the soft-rot symptom in the mesophyll space of tobacco leaf (Fig. 5). These results imply that the enrichment of PFLP in mesophyll space of tobacco leaf would inhibit soft-rot symptom cause by *E. carotovora*.

The resistance inducing in full-length *pflp* transgenic tobacco was due to activation of HCD [8]. However neither soft-rot symptom nor hypersensitive cell death (HCD)-like necrosis was observed in the d-*pflp* transgenic tobacco after inoculation of *E. carotovora* (Fig. 6d). In the initial period of infection, the bacterial multiplication in the d-*pflp* transgenic tobacco was inhibited (Fig. 6e) but not in the full-length *pflp* transgenic tobacco. We suppose the different result between d-*pflp* and full-length *pflp* transgenic tobacco is due to the distribution of the heterologous Fd. In the full-length *pflp* transgenic tobacco, most abundant PFLP might be travelled to chloroplast as much as they can however the abundant PFLP over expressing in the d-*pflp* transgenic tobacco was stacked in the cytoplasm only. This difference might result to pathogen more difficult to get iron from debris of d-*pflp* transgenic tobacco than full-length *pflp* transgenic tobacco. Therefore, the pathogen would lose the opportunity to cause HCD-like necrosis or soft-rot symptom in the d-*pflp* transgenic tobacco.

In summary, we demonstrated that PFLP exhibits an anti-microbial activity in vitro and in vivo. The anti-microbial activity of PFLP seems to be depending on the iron-binding capability. Furthermore, abundant PFLP would inhibit symptom caused by bacterial pathogen in the mesophyll space of tobacco leaf. The d-*pflp* transgenic tobacco over expressing abundant PFLP in the cytoplasm has against infection of *E. carotovora* without induction of HCD-like necrosis.

Acknowledgment

This work was supported by grants to T.Y. Feng from Academia Sinica, Taiwan, ROC.

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