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Purification of flagellin of *Pseudomonas fluorescens* GcM5-1A carried by the pine wood nematode, *Bursaphelenchus xylophilus*, and its *in vitro* toxicity to a suspension of cells of *Pinus thunbergii*

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Summary. A 50 kDa protein was isolated from a culture of *Pseudomonas fluorescens* strain GcM5-1A, carried by pine wood nematode, *Bursaphelenchus xylophilus*. The protein was purified through ammonium sulphate precipitation, DEAE-Sepharose FF ion exchange chromatography and a Superdex-75 column. N-terminal sequence alignment showed high homology to the flagellin of *P. fluorescens* strain Pf-5. Meanwhile, the gene encoding flagellin of strain Pf-5 was cloned and overexpressed in *Escherichia coli* and the recombinant flagellin was purified to homogeneity by chromatography on a nickel resin column followed by MonoQ ion-exchange chromatography. The antiserum raised against flagellin of strain Pf-5 showed strong reaction with the 50 kDa protein, suggesting that the protein from strain GcM5-1A was flagellin. Bioassay results demonstrated toxicity of flagellins from both strains to a suspension of cells of the Japanese black pine, *Pinus thunbergii*. Cells treated with flagellin shrank markedly as a result of cytoplasm condensation, indicating the possible occurrence of programmed cell death.

Key words: *Bursaphelenchus xylophilus*, flagellin, *Pinus thunbergii, Pseudomonas fluorescens,* purification.

The pine wood nematode (PWN), *Bursaphelenchus xylophilus*, is the direct cause of pine wilt disease. PWN is native to North America and was introduced to Japan through the timber trade, from where it has been spread into China and Korea. The nematode was first reported in Portugal in 1999 (Mota *et al.*, 1999) and nowadays it has become a world-wide threat to forest resources. To find a solution to control completely the pine wilt disease, it is essential to understand the mechanism of how PWN causes the disease.

For a long time, the nematode was thought to be the sole pathogen of pine wilt disease (Mamiya, 1983; Myers, 1988). However, in recent years, increased evidences suggested that bacteria associated with PWN were involved in pathogenesis (Oku *et al.*, 1980; Kawazu & Kaneko, 1997). Each nematode from naturally infected Japanese black pines, *P. thunbergii*, carried an average of 290 bacterial cells and bacteria on the surface of PWN could be seen clearly under the electron microscope (Guo *et al.*, 2002; Zhao *et al.*, 2006). A survey of species of bacteria carried by PWN isolated from diseased *P. thunbergii* and *P. massoniana* in China indicated that 24 bacteria strains were found on the surface of PWN. Bioassays showed that 17 of the 24 strains could produce phytotoxins, and most strains belonged to genus *Pseudomonas* (Zhao *et al.*, 2003). Previous experiments indicated that inoculation of aseptic *B. xylophilus* did not lead to browning of callus or wilt of aseptic Japanese black pine seedlings. However, seedlings inoculated with aseptic nematodes plus *P. fluorescens* showed severe symptoms. In addition, the filtered culture of *P. fluorescens* could induce similar symptoms to Japanese black pine seedlings and callus as wild PWN, suggesting that pine wilt disease was caused by co-infection of PWN and the bacteria it carried (Han *et al.*, 2003). The pathogenesis of the pine wilt disease could be due partially to the secretion of toxins from the bacteria. Guo *et al.* (2007)

isolated and identified two phytotoxins, cyclo (-Pro-Val-) and cyclo (-Pro-Tyr-), from the culture of *P. fluorescens* GcM5-1A, and the two compounds showed relatively strong toxicities to both suspension cells and seedlings of Japanese black pine *in vitro* (Guo *et al.*, 2007). Besides the small molecular toxins, proteins precipitated with ammonia sulphate from the culture of bacteria carried by PWN also demonstrated strong toxicity to suspension of cells of Japanese black pine. To further elucidate the role of bacteria carried by PWN in pine wilt disease, especially the role of proteins secreted by these bacteria, here we report the isolation and identification of a 50 kDa protein produced by *P. fluorescens* GcM5-1A, and studies on its toxicity *in vitro* to suspension of cells of *P. thunbergi*.

MATERIAL AND METHODS

Plant and bacterial strains. Seeds of Japanese black pine were collected in Zhongshan Park, Nanjing, China. *P. fluorescens* GcM5-1A was isolated and identified from PWN in diseased *P. thunbergii* in China as described by Zhao *et al.* (2003).

Bacteria culture. Single colony of *P. fluorescens* GcM5-1A was inoculated into 30 ml LB medium, and cultured in a shaking incubator at 30°C for 24 h. The culture was then inoculated into 2 l LB medium in an 8 l flask and cultured in the same way as above. Cells were harvested by centrifugation at 15,000 g for 20 min at 4°C and the bacteria-free supernatant was collected for isolation of toxic proteins.

Purification of protein toxins from *P. fluorescens* GcM5-1A. For purification of the protein toxins, ammonium sulphate was added to the supernatant to a saturation of 20%. After the precipitated proteins were collected by centrifugation at 4°C and 16 000 g for 30 min, ammonium sulphate was added to the supernatant until saturation of 50%. The precipitated proteins were then collected in the same manner as described above. Pellets of the two saturations were dissolved in distilled water and the suspensions were desalted by dialysis against distilled water before bioassays. For purification of flagellin, the pellet from 20% ammonium sulphate saturation was resuspended in 50 ml TE buffer (20 mM Tris-HCl, pH 8.0, 5 mM EDTA) and the resultant solution was dialyzed against TE buffer before being loaded onto a DEAE-Sepharose FF column (1.2×10 cm). Proteins were eluted with a NaCl gradient (0.1-0.5 M) and fractions containing the 50 kDa protein were pooled and concentrated by a Amicon ultrafiltration tube before further purified on a Superdex-75 column (1.6×60 cm) and elution with TE buffer. Purity of the protein was analyzed by SDS-PAGE and concentration of the purified protein was determined by the Bradford method (Bradford, 1976), using bovine serum albumin as the standard.

N-terminal sequencing of the purified flagellin. The purified protein with a molecular weight of 50 kDa was separated by SDS-PAGE with a 12% polyacrylamide gel. Proteins were then electro-transferred onto a PVDF membrane and the 50 kDa band was excised after the membrane was stained with 0.1% Coomassie Brilliant Blue R250 in 40% methanol supplied with 1% acetic acid and destained with 50% methanol. N-terminal sequencing was performed with ABI 473 (Applied Biosystems, USA).

Overexpression of recombinant flagellin from *P. fluorescens* Pf-5 in *Escherichia coli*. The *fliC* was synthesized in TaKaRa biotechnology (Dalian Co. Ltd) according to the published sequence (GenBank accession № NC004129, Region: 1832150 -1833001) of *P. fluorescens* Pf-5 (Gross *et al.*, 2005) with a *Nde* I site at 5'end and a *Xho* I site at 3'end, respectively. The DNA fragment was cloned into pMD-18T and verified by sequencing.

To construct the expression vector pET-15fliC, fliC in pMD-18T plasmid was digested with *Nde* I and *Xho* I, and then ligated with the linearized pET-15b vector digested with the same endonucleases. The expressing vector was transformed into *E. coli* BL21 (DE3) following the standard protocol. The engineered bacterium was then cultured overnight in 100 ml LB medium supplemented with 100 μg ml^{-1} ampicillin at 37°C in a shaking incubator. Later, the culture was transferred to 2 l of the same medium and culturing was continued for 5 h under the same conditions. Protein expression was induced for 8 h by dropping the culturing temperature to 28°C and adding 0.5 mM isopropyl-β-D-thiogalacto-pyranoside (IPTG). Finally, cells were harvested by centrifugation at 10 000 g and 4°C for 10 min.

Purification of recombinant flagellin. Cell pellets expressing *fliC* were resuspended in 50 ml binding buffer (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 5 mM imidazole) and sonicated for 20 times at 0°C, 10 s each time with a 60 s interval. Cell lysis was achieved by centrifugation at 15,000 *g* and 4°C for 30 min. After the supernatant was passed through a nickel column (1.6×8 cm), the column was washed with 50 ml binding buffer followed by 25 ml washing buffer (20 mM Tris-HCl, pH8.0, 0.5 M NaCl, 100 mM imidazole). Recombinant

flagellin was eluted with 25 ml elution buffer (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 200 mM imidazole). The elute was concentrated to 5 ml with a concentrator (PALL Corp., USA) and then diluted to 50 ml with TE buffer. Later, the protein solution was applied to a MonoQ column (GE Healthcare, USA) and proteins were eluted by a linear NaCl gradient in TE buffer. Purity of the recombinant flagellin was analyzed by SDS-PAGE.

Culturing of aseptic Japanese black pine callus. Culturing of the pine callus followed the procedure of Zhao *et al.* (2003). Briefly, calli of Japanese black pine were induced on the tissue culture medium (1/2MS + 2,4-D 10.0 mg l^{-1} + KT 4.0 mg l^{-1} + 6-BA 4.0 mg l^{-1}) from mature embryos of *P. thunbergii* at 27°C in the dark. The callus was then subcultured on the calli maintaining medium (1/2 MS + NAA 0.5 mg l^{-1} + IBA 0.1 mg l^{-1} + GA_3 1.0 mg l^{-1} + LH 100 mg l^{-1} + 2,4-D 1.0 mg l^{-1}) under the same conditions as described above.

Toxicity of flagellins to suspension cells of *P. thunbergii*. The purified flagellins from both *P. fluorescens* GcM5-1A and *P. fluorescens* Pf-5 were dialyzed overnight against 2 l of distilled water. The desalted protein (0.1 ml) was added to 0.4 ml suspension cells of *P. thunbergii* in 1/2 MS medium to a final concentration of 100 μg l^{-1}. As a negative control, an equal volume of distilled water was added to the same suspension cells. Cells were incubated at room temperature for 5 min before stained with fluorescein diacetate (FDA) and examined under a fluorescence microscope. Lethal rate was calculated according to the method of Zhao *et al.* (2003).

Preparation of antiserum against Pf-5 flagellin. Three Japanese adult male rabbits were simultaneously immunized with a mixture of 200 μg flagellin from *P. fluorescens* Pf-5 and the same volume of complete Freund's adjuvant (Yang *et al.*, 2003). Three weeks later, these rabbits were further immunized with a mixture of, 100 μg flagellin and the same volume of incomplete Freund's adjuvant. Blood serum was prepared from the blood of rabbit carotid arteries after 10 days.

Western blot analysis. Five microlitre of 1.0 μg flagellin solution from strain GcM5-1A was mixed with the same volume of 2×SDS-PAGE loading buffer and boiled for 5 min. After the sample was separated by SDS-PAGE with a 12% polyacrylamide gel, flagellin was transferred onto a polyvinyldifluoridine (PVDF) membrane (PALL Corporation, USA). The lane for protein marker was cut down and stained with 0.1% Coomassie Brilliant Blue R250 in 40% methanol. Western blot analysis was processed according to the standard protocol using antiserum against the Pf-5 flagellin and HRP-labelled sheep anti-rabbit IgG. Protein bands were visualized using the substrates 3,3'-diaminobenzidine and H_2O_2 (Yang *et al.*, 2003).

Effects of flagellin on morphology of suspension cells. Pine calli (approximate 0.1 g) were suspended in 0.6 ml 0.67×MS liquid medium and 0.2 ml flagellin was added to a final concentration of 100 μg ml^{-1}. As a control, 0.2 ml distilled water was added to the same volume of suspension cells. Suspension cells treated with or without flagellin in 1/2 MS medium were incubated for 48 h at room temperature. The cell morphology was observed under a light microscope.

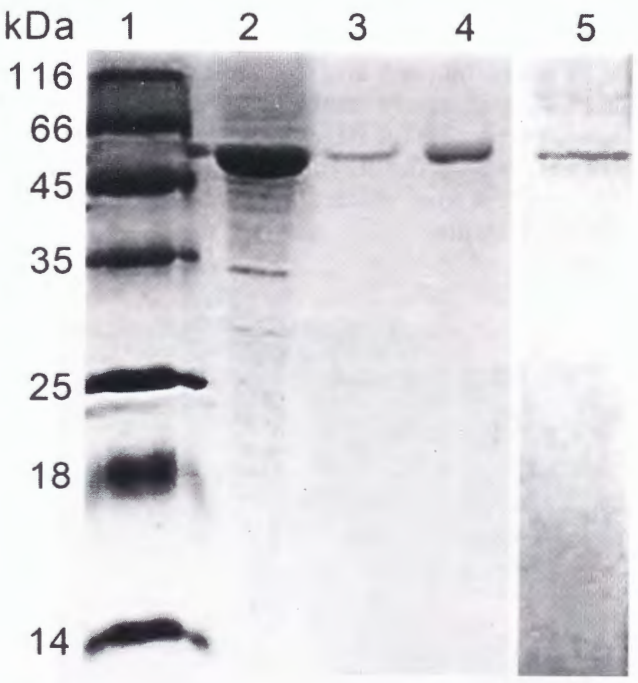

Fig. 1. Purification and Western blot analysis of flagellin from culture of *P. fluorescens* GcM5-1A. Lane 1, protein marker; 2, total protein from 20% ammonium sulfate precipitation; 3, sample eluted from Superdex-75 column; 4, sample from DEAE-Sepharose FF column; 5, Western blotting analysis of the purified flagellin from *P. fluorescens* GcM5-1A.

RESULTS

Isolation and identification of flagellin secreted by P. fluorescens GcM5-1A. GcM5-1A cells were cultured in LB medium and proteins in the cell-free culture were precipitated by ammonium sulphate of different saturations. After dialysis, both of the crude proteins precipitated by

ammonium sulphate of 20% and 50% saturations showed toxicity to cells of Japanese black pine (data not shown). The precipitation of 20% saturation contained a large amount of 50 kDa protein (Fig. 1). After further separations by chromatography on DEAE-Sepharose FF column and on Superdex 75 column, the 50 kDa protein was purified to homogeneity as inferred by SDS-PAGE (Fig. 1). N-terminal sequence analysis suggested the first ten amino acid residues to be ALSVNTNITS, with the start codon encoded M removed. Sequence alignment showed that the N-termini was homologous to the N-terminal sequence of flagellin from *P. fluorescens* Pf-5, with the fourth serine residue substituted by a similar threonine and the ninth isoleucine residue replaced to a similar hydrophobic valine residue in Pf-5.

Western blotting analysis indicated that the 50 kDa protein could react with antiserum raised against recombinant flagellin of *P. fluorescens* Pf-5 (Fig.1), which further demonstrated that the 50 kDa protein from culture of *P. fluorescens* GcM5-1A was flagellin.

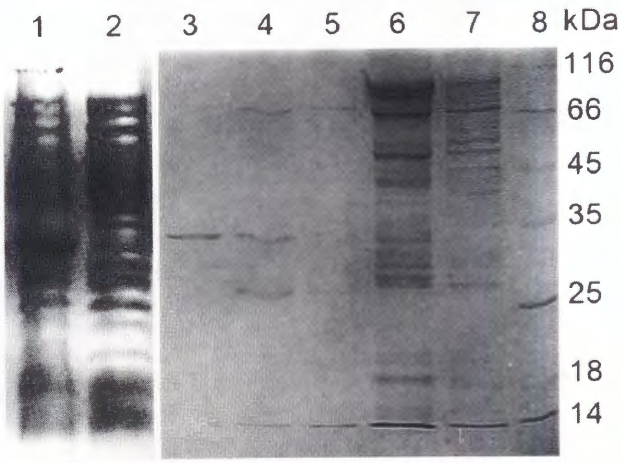

Fig. 2. Overexpression and purification of recombinant *P. fluorescens* Pf-5 flagellin produced in *E. coli*.

Lane 1, total proteins from *E. coli* BL21(DE3) harboring pET-15bfliC; 2, total proteins from untransformed *E. coli* BL21(DE3); 3, flagellin eluted from MonoQ column; 4, proteins eluted from nickel column with 200 mM imidazole; 5, proteins eluted with washing buffer; 6, supernatant of IPTG induced engineered bacteria lysate; 7, supernatant from untransformed *E. coli* BL21(DE3); 8, protein marker.

Overexpression and purification of flagellin from *P. fluorescens* Pf-5 in *E. coli*. Gene *fliC* encoding flagellin of *P. fluorescens* Pf-5 was cloned into expression vector pET-15, under the control of T7 promoter. Recombinant flagellin was overexpressed in *E. coli* BL21 (DE3) induced by IPTG (Fig. 2). Cells of the engineered bacteria were lysed and proteins from different fractions were analyzed by SDS-PAGE. The recombinant flagellin was found mainly in inclusion bodies, even though the culturing temperature had been cooled to 28℃. Only part of flagellin was left in the supernatant as soluble form, and further purification was processed from this fraction.

The recombinant flagellin in the supernatant was first partially purified by affinity chromatography on a nickel column based on its N-terminal His-tag. Since there were still contaminant proteins after nickel column chromatography as analyzed by SDS-PAGE, further purification was conducted using MonoQ ion exchange chromatography to homogeneity. The purified protein had a molecular weight of 33 kDa, consist with that inferred from its coding gene *fliC* (Fig. 2).

Toxicity of flagellins to suspension cells of *P. thunbergii*. To investigate the toxicity of flagellins to cells of *P. thunbergii in vitro*, cells were treated with either the purified flagellin from GcM5-1A or the recombinant flagellin from Pf-5 for different time periods. Cells were then stained with FDA and observed under a fluorescent microscope. Lethal rates were calculated and results indicated that both flagellins exerted similar toxicity to the cells of *P. thunbergii*. The lethal rate of cells treated with 100 μg ml^{-1} flagellin from *P. fluorescens* GcM5-1A for 4 days reached 91%, compared with 49% in the control cells in distilled water. The lethal rate of suspension cells treated with 100 μg ml^{-1} flagellin from *P. fluorescens* Pf-5 was 94% on the fourth day (Fig. 3). Data also indicated that the lethal rate increased proportionally to the flagellin concentration and length of the treatment time.

Effects of flagellin on morphology of suspension cells. Cells of Japanese black pine treated with flagellin of *P. fluorescens* GcM5-1A began to necrose on the first day, as indicated by the weak fluorescence signal after FDA staining (Fig.4 A, B). Forty-eight hours later, the morphology of cells in the treated group was different from that of cells in the control group. The flagellin treated cells were shrunken with condensed cytoplasm and nuclei became marginal, all of which were important morphological characteristics of programmed cell death. Cells in the control group, however, had plasma membranes closely attached to cell walls, and there were many oil drop-like granules in the cytoplasm, indicating that these cells were in a healthy conditions (Fig.4 C, D).

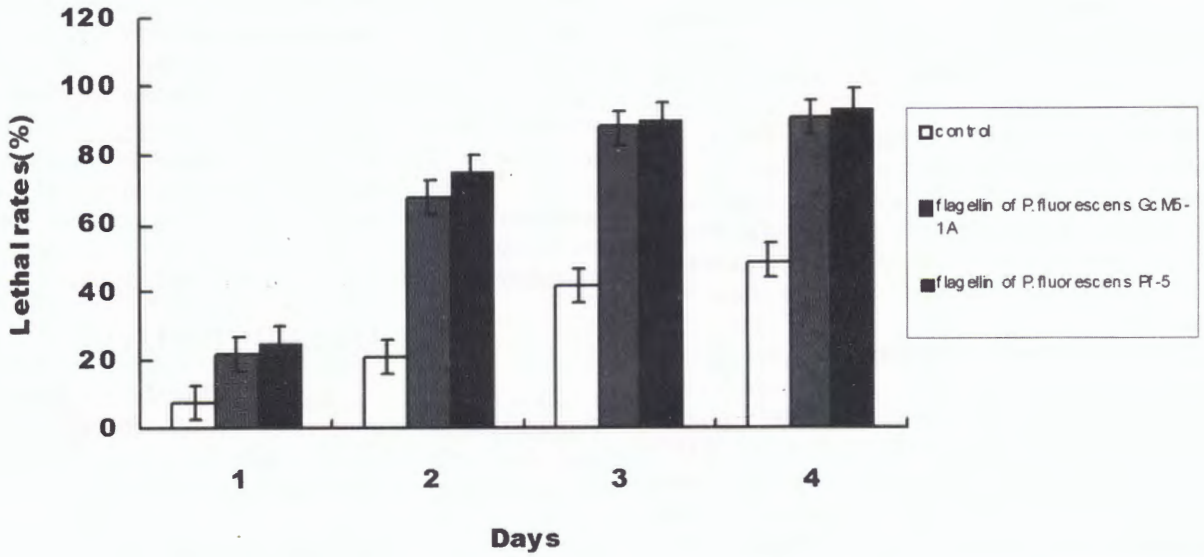

Fig. 3. Toxicities of flagellins from *P. fluorescens* GcM5-1A and *P. fluorescens* Pf-5.

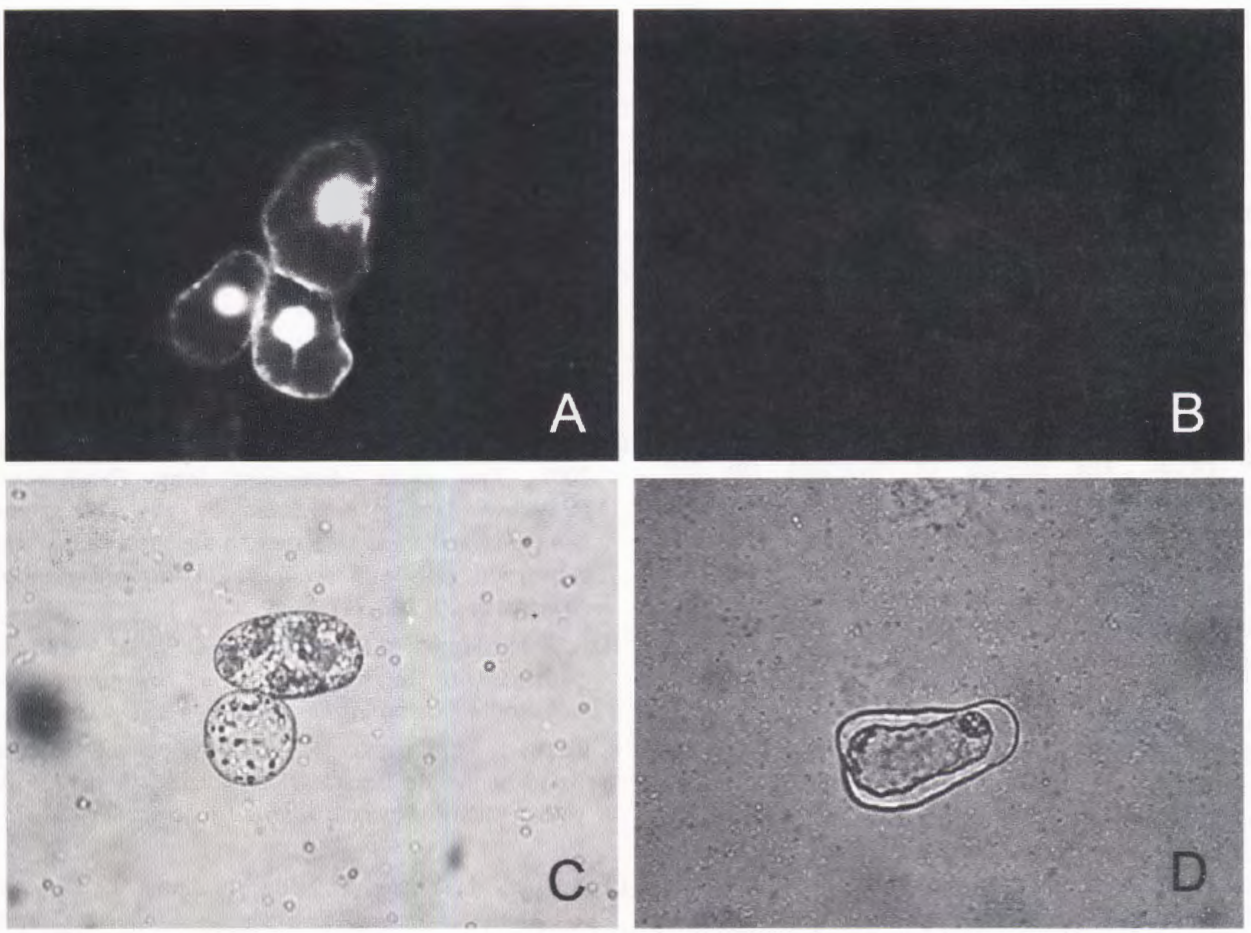

Fig. 4. Morphological differences of *P. thunbergii* suspension cells treated and untreated with flagellin.

A: Control cells appeared healthy with strong green fluorescence; B: flagellin treated cells began to necrose within 24 h; C: Cytoplasm of control cells appeared normal; D: cytoplasm of flagelin treated cells was condensed and nuclei were translocated to the margin of cell.

DISCUSSION

Previous studies suggested that pine wilt disease was induced by both PWN and its associated bacteria (Zhao *et al.*, 2003). To investigate the roles of the bacteria in pine wilt disease, one typical bacterial strain carried by PWN, *P. fluorescens* GcM5-1A, was chosen as a test bacterium and its secreted toxic proteins were studied *in vitro*. A protein with a molecular weight of 50 kDa, which was purified and identified as flagellin, was found to be toxic to suspension cells of *P. thunbergii*.

Flagellin, the main building block of the bacterial flagellum, acts as a pathogen-associated molecular pattern to trigger the innate immune response in animals and plants (Li *et al.*, 2005). However, flagellins from different bacteria might have different effects on plant cells. Flagellins purified from *P. syringae* pv. *tomato* and *P. s.* pv. *glycinea* (both are incompatible pathogens to tobacco plants) induced fragmentation of chromosomal DNA and an oxidative burst accompanied by programmed cell death in tobacco cells. On the other hand, flagellin from *P. syringae* pv. *tabaci*, a compatible pathogens, did not have the same effect (Taguchi *et al.*, 2003). Besides the typical characteristics of programmed cell death induced by flagellins, Tanaka *et al.* (2003) reported that flagellin from an incompatible strain of *Acidovorax avenae* induced rapid H_2O_2 generation accompanying hypersensitive cell death, and the flagellin-deficient incompatible strain lost this ability. Another study demonstrated that flagellin induced basal resistance in the leaves of *Nicotiana benthamiana*, accompanied by reduced vascular flow into minor veins (Oh & Collmer, 2005). In this paper, the effects of flagellin on the suspension cells of *P. thunbergii*, a species of *Gymnospermae*, was investigated for the first time. It was observed that the cytoplasm of suspension cells treated with flagellin became condensed and cells became shrunken, indicating that flagellin might have induced programmed cell death.

Taguchi *et al.* (2005) reported that flagellin from *P. syringae* pv. *tabaci* 6605 was glycosylated and this glycosylation was essential for bacterial virulence. In this paper, we found that the relative molecular weight of flagellin from *P. fluorescens* GcM5-1A was much larger than that of flagellin from *P. fluorescens* Pf-5. However, both flagellins from the two different strains showed similar toxicity to suspension cells of Japanese black pine. It is likely, although still unclear, that the increased molecular weight of flagellin of *P. fluorescens* GcM5-1A could be due to glycosylation. Results presented in this paper, as well as several other studies, clearly demonstrated that flagellins of bacteria carried by PWN played certain roles in pine wilt disease. On the one hand, flagellins could induce a basal immune response to resist the invasion of the plant pathogen. On the other hand, flagellins might induce cell death and inhibit water transportation, resulting in the symptom of wilt. Further biochemical and biological studies on flagellin are needed to elucidate the mechanism of pine wilt disease.

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Guo D.,, Zhao B, Li R, Kulinich O. A., Ryss A. Очистка флагеллина бактерий *Pseudomonas fluorescens* GcM5-1A переносимых нематодой сосны *Bursaphelenchus xylophilus*, и его токсичность *in vitro* для суспензий клеток *Pinus thunbergii*.

Резюме. Белок весом 50 kDa выделен из культуры бактерий *Pseudomonas fluorescens* штамма GcM5-1A, переносимых нематодой сосны *Bursaphelenchus xylophilus*. Белок был очищен с помощью преципитации в сульфате аммония, DEAE-Sepharose FF ион-обменной хроматографии и колонок Superdex-75. N-терминальные последовательности белка показывают высокий уровень гомологии с флагеллином *P. fluorescens* штамма Pf-5. Гены, кодирующие флагеллин штамма Pf-5, были клонированы и экспрессированы в *Escherichia coli*, а рекомбинантный флагеллин был очищен до гомогенности хроматорафией на никелево-резиновых колонках, с последующей ион-обменной MonoQ хроматографией. Антисыворотка против флагеллина штамма Pf-5 показала высокий уровень реактивности с выделенным белком в 50 kDa, что позволило предположить, что этот протеин из штамма GcM5-1A также является флагеллином. Биологическое тестирование показало, что флагеллины из обоих штаммов были токсичны для суспензии клеток сосны *Pinus thunbergii*. Клетки, обработанные флагеллинами, заметным образом сжимались к результате конденсации цитоплазмы, указывая на возможную программируемую клеточную смерть.