A novel antimicrobial protein isolated from potato (*Solanum tuberosum*) shares homology with an acid phosphatase

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A novel antimicrobial protein (AP_1) was purified from leaves of the potato (*Solanum tuberosum*, variety MS-42.3) with a procedure involving ammonium sulphate fractionation, molecular sieve chromatography with Sephacryl S-200 and hydrophobic chromatography with Butyl-Sepharose using a FPLC system. The inhibition spectrum investigation showed that AP_1 had good inhibition activity against five different strains of *Ralstonia solanacearum* from potato or other crops, and two fungal pathogens, *Rhizoctonia solani* and *Alternaria solani* from potato. The full-length cDNA encoding AP_1 has been successfully cloned by screening a cDNA expression library of potato with an anti-AP_1 antibody and RACE (rapid amplification of cDNA ends) PCR. Determination of the nucleotide sequences revealed the presence of an open reading frame encoding 343 amino acids. At the C-terminus of AP_1 there is an ATP-binding domain, and the N-terminus exhibits 58 % identity with an/the acid phosphatase from *Mesorhizobium loti*. SDS/PAGE and Western blotting analysis suggested that the AP_1 gene can be successfully expressed in *Escherichia coli* and recognized by an antibody against AP_1. Also the expressed protein showed an inhibition activity the same as original AP_1 protein isolated from potato. We suggest that AP_1 most likely belongs to a new group of proteins with antimicrobial characteristics *in vitro* and functions in relation to phosphorylation and energy metabolism of plants.

Key words: antimicrobial protein, acid phosphatase, potato.

INTRODUCTION

Plants protect themselves against microbial pathogens by various defence responses, which include the production of antimicrobial peptides, secondary metabolites, lytic enzymes, membrane-interacting proteins and reinforcement of cell walls [1,2]. During the past few years a wide spectrum of plant antimicrobial proteins has been detailed, and enhanced resistance has been obtained by introducing the corresponding genes into crop species to produce transgenic lines [3]. The most intense research focuses on elucidation of the subsequent signalling pathways that govern the activation of genes encoding antimicrobial proteins. To date, most antimicrobial proteins that have been identified belong to a group of small molecular mass antimicrobial peptides [4,5]. Several families of antimicrobial peptides have been characterized in plants. The majority of them have a cysteine-rich structure, and a number of them show both constitutive- and pathogen-inducible expressions [6–8].

Bacterial wilt caused by *Ralstonia solanacearum* [8a], is one of the most important and widespread bacterial diseases of plants in the world. Over the past decade, attempts have been made to transfer some foreign genes, such as Cf-9 and Ve (tomato, *Lycopersicon esculentum*) [9,10], chitinase gene (from the fungus *Trichoderma harzianum*) [11], defensins (alfalfa, *Medicago sativa*) [12], H_2O_2-generating enzymes (from the fungus *Aspergillus niger*) [13], antimicrobial peptides (insects) [14] and antiviral protein II (pokeweed, *Phytolaccaceae acinosa*) into potato (*Solanum tuberosum*). Only a few genes from potato, such as snakin-1 and snakin-2, were reported to be able to increase resistance to bacterial diseases [15,16].

In previous studies, we found a phenomenon that crude protein extracts from leaves of bacterial wilt-resistant potato germ plasms possess microbial inhibition activity. In the present paper, we will report the purification and cloning of this antimicrobial protein (AP_1). The expression of AP_1 cDNA in *Escherichia coli* cells and the detection of inhibition activity has also been studied.

EXPERIMENTAL

Biological materials

A bacterial wilt-resistant variety (MS-42.3) of potato plants were grown in a greenhouse at 18–25 °C in a 12-cm pot containing peat/vermiculite (3:1). Leaves were harvested from 7-leaf-stage seedlings. Five strains of *R. solanacearum* isolated from different host plants, PO_{41} (potato, race 3), PO_1 (potato, race 1), TM_1 (tomato, race 1), TB_{23} [tobacco (*Nicotiana tabacum*), race 1] and P_8 [groundnut (*Arachis hypogaea*), race 1] were cultured as described by He et al. [17]. Two fungal pathogens from potato, *Rhizoctonia solani* and *Alternaria solani*, were cultured on PDA medium [20 % (w/v) potato, 1.5 % (w/v) glucose and 1.5 % (w/v) agar].

Abbreviations used: cfu, colony-forming units; IPTG, isopropyl β-D-thiogalactoside; RACE, rapid amplification of cDNA ends; UspA, universal stress protein A.

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The nucleotide and amino acids sequences for AP_1 will appear in the GenBank® and NCBI databases under accession number AY297449.

Purification of potato AP_1

The crude proteins were obtained as following: potato leaves of the MS-42.3 variety (800 g) were ground and incubated with extraction buffer containing 25 mM Tris/HCl (pH 8.0) at 4 °C for 1 h. The homogenate was squeezed through gauze and clarified by centrifugation for 25 min at 10 000 ***g***. Solid ammonium sulphate was add to the supernatant to obtain 40 % relative saturation, and the precipitate formed after standing for 1 h at 4 °C was removed by centrifugation at 10 000 ***g*** for 25 min. The supernatant was adjusted to 70 % relative ammonium sulphate saturation, and the precipitate was allowed to form overnight at 4 °C. The precipitate was collected by centrifugation at 10 000 ***g*** for 25 min at 4 °C, and was then redissolved in 200 ml of distilled water, and the insoluble material was removed by further centrifugation at 10 000 ***g*** for 25 min. The clear supernatant was dialysed extensively against distilled water for 2 days to remove salt. After dialysis, the solution was concentrated for detecting inhibition activity on the indicator strain PO_{41} of *R. solanacearum*. The solution was adjusted to 25 mM Tris/HCl (pH 8.0) by addition of the 10-fold-concentrated buffer, and subsequently passed over a molecular sieve chromatography column (2.6 cm × 60 cm) of Sephacryl S-200 equilibrated with 25 mM Tris/HCl (pH 8.0). The proteins were eluted with the same buffer at a flow rate of 36 ml/h. The fractions with inhibition activity against bacteria were pooled and purified further using a FPLC system with a hydrophobic Butyl-Sepharose (LKB-Pharmacia, Uppsala, Sweden). The column (vol., 1 ml) was equilibrated with buffer B (50 mM phosphate buffer, pH 7.0) for 5 min, and then with buffer A (2.0 M ammonium sulphate/50 mM phosphate buffer, pH 7.0) for 10 min. The pooled fractions were loaded on to a column and washed with buffer A, and then the proteins were eluted with linear gradient of 2.0–0.0 M ammonium sulphate at a flow rate of 30 ml/h. The fractions containing activity of interest were pooled, dialysed and concentrated to dry powder. Proteins were dissolved in distilled water for bacterium inhibition tests and SDS/PAGE. Before inhibition tests, all protein samples were sterilized by filtration through a 0.22-μm filter in order to avoid contamination.

Bacterial inhibition activity detection

Inhibition activities of AP_1 against *R. solanacearum* strains PO_{41}, PO_1, TM_1, TB_{23} and P_8 were tested in plates by mixing 100 μl of bacterial suspension [final concentration 5×10^5 cfu (colony-forming units)/ml] in nutrient agar medium. Protein sample (50 μl, 2.5 μg) was injected into 6-mm-diameter wells. The plates were incubated at 30 °C and inhibition zones were measured after 48 h.

Antifungal activity assays

The agar-well diffusion method was used to detect antifungal activity. All experiments assaying inhibitory activity against *R. solani* and *A. solani* were performed in duplicate. Agar plugs 5 mm in diameter were taken from *R. solani* or *A. solani* cultures, and transferred to the centre of plates. The plates were incubated for 24–72 h at 30 °C. Protein sample (50 μl, 2.5 μg) was injected into 6-mm wells, approx. 1 cm from the edge of the fungal colonies. The plates were incubated at 30 °C and inhibition effect was observed after 30–72 h.

Preparation of anti-AP_1 serum

Anti-AP_1 antigen was purified using a FPLC system with a hydrophobic Butyl-Sepharose (Pharmacia-LKB). A New Zealand rabbit was immunized intramuscularly with antigen solution containing 200 μg of AP_1 emulsified in Freund's complete adjuvant, as described by Sambrook et al. [18].

Potato AP_1 cDNA cloning

To clone the AP_1 gene, mRNA from the MS-42.3 variety potato leaves was extracted by using PolyATtract mRNA Isolation System (Promega). The cDNA construction and screening were carried out according to the manufacturer's instructions. The cDNA expression library (approx. 1×10^5 plaque-forming units) constructed in λgt11 vector was screened with anti-AP_1 antibody. Antigen–antibody–alkaline-phosphatase complexes were located using the substrate 5-bromo-4-chloroindol-3-yl phosphate in combination with Nitro Blue Tetrazolium. The positive plaques appeared an intense purple colour at the site of the antigen–antibody complexes. The positive clones obtained were purified and the sizes of their cDNA inserts were analysed. After subcloning in pGEM vectors (Promega), the cDNA inserts were sequenced. All of cDNAs of interest obtained from expression cDNA library did not contain 5′ upstream regions.

To obtain the full-length cDNA, RACE (rapid amplification of cDNA ends) PCR was carried out using MARATHON cDNA Amplification kit (ClonTech). The primers at the 5′-end (5′-CCATCCTAATACGACTCACTATAGGGC-3′, provided by the manufacturer) and 3′-end (5′-TCTAGAGTAGAAGCGAATGTTCCG-3′, designed on the basis of the known 3′-end sequence of AP_1 cDNA) were used in the amplification. The product from the RACE PCR was cloned into the pGEM-T Easy vector. To join the 5′-end and 3′-end of cDNA fragments together, PCR was performed with primers (5′-GGATCCATGCAACCGTCGGCCAGCGAC-3′ and 5′-GAATTCCACACGCCATACTTAAAACCC-3′) according to the manufacturer's instructions (ClonTech), and *Bam*HI and *Eco*RI sites were introduced in the 5′- and 3′-ends of the full length of AP_1 during the PCR amplification. The double direction sequencing was completed with T_7 and SP_6 primers in pGEM-T Easy vector.

Construction of AP_1 over-expression plasmid

Potato AP_1 was expressed in *E. coli* as a fusion protein, the coding sequence of AP_1 cDNA was cut from pGEM-T Easy vector with *Bam*HI and *Eco*RI. The full-length AP_1 cDNA was inserted into pET-5a vector between *Bam*HI and *Eco*RI sites. The resulting plasmid was firstly introduced in *E. coli* JM109 strain, and then pET-5a-AP1 recombinant was transformed into expression strain *E. coli* BL21(DE3) pLysS.

Expression of AP_1 in *E. coli* and the production of fusion protein

To produce the AP_1-fusion protein, 10 ml of an overnight culture of *E. coli* BL21(DE3) containing pET-5a-AP1 was used to inoculate 1 litre of Luria–Bertani broth supplemented with 0.01 % (w/v) ampicillin. A culture containing no AP_1 construct incubated under the same conditions was used as a control. After incubation at 37 °C for 2.5 h, 0.4 mM IPTG (isopropyl β-D-thiogalactoside) was added and incubation was continued for 3 h. Cells were harvested by centrifugation, washed once with buffer C [50 mM Tris/HCl (pH 8.3) and 10 mM 2-mercaptoethanol], and the pellet was resuspended in 3 ml of lysis buffer [50 mM Tris/HCl (pH 8.0), 1 mM EDTA and 100 mM NaCl] and disrupted by sonication. The cell lysate was collected by centrifugation at 12 000 ***g*** for 15 min at 4 °C, then the supernatant was removed and pellet was resuspended in 100 μl of water. Equal aliquots

of soluble and insoluble fractions were analysed by SDS/PAGE to determine if most of the AP_1-fusion protein was in the pellet. Because full-length AP_1 was always found in the insoluble fraction, various modifications of the standard protocol were attempted to achieve production of AP_1 in soluble form. The inclusion bodies containing AP_1-fusion protein were dissolved in 100 μl of lysis buffer with 6 M urea and 0.1 mM PMSF. The fusion samples were further concentrated for SDS/PAGE and inhibition activity tests.

SDS/PAGE and Western blot analysis

Protein samples were subjected to SDS/PAGE (12 % gel), as described in [18,19], and were then electrotransferred on to a nitrocellulose membrane at 600 mA at 4 °C for 3 h. The membrane was blocked in 25 mM Tris/HCl (pH 7.4) containing 0.8 % (w/v) NaCl, 0.02 % KCl (TBS buffer) and 5 % (w/v) non-fat dried milk for overnight incubation at 4 °C, and then probed with purified anti-AP_1 serum at 1:1000 (in TBS buffer) for 2 h at 4 °C. The membrane was washed three times, 15 min each time, with TBS buffer containing 0.1 % (v/v) Tween 20, and then incubated for 1 h in the same buffer containing goat anti-rabbit IgG–alkaline phosphatase conjugate diluted to 1:7500, and subsequently washed three times in TBS/Tween 20 buffer. The reaction was visualized by immersion in a developing solution [10 ml of 100 mM Tris/HCl (pH 9.5), 5 mM $MgCl_2$, 100 mM NaCl, 1.6 mg of 5-bromo-4-chloroindol-3-yl phosphate and 3.2 mg of Nitro Blue Tetrazolium].

DNA sequencing and analysis

The DNA sequences were determined with a sequencing kit (Dye-labelled Terminators, PerkinElmer). The sequencing reactions were resolved on an ABI377 automated sequencer (PerkinElmer). DNA homology searches and the deduced amino acid sequences were performed using the NCBI BLAST server.

RESULTS

Purification of potato AP_1

AP_1 was purified by a three-step procedure. The first step involved ammonium sulphate fractionation at 40–70 % relative saturation. After ammonium sulphate precipitation, the inhibition activity against *R. solanacearum* strain PO_{41} was detected. In the second step, the proteins were purified by molecular sieve chromatography on a Sephacryl S-200 column, and all of fractions with antibacterial activity were further separated in the third step using an FPLC system with hydrophobic Butyl-Sepharose. Elution of the column with 2.0–0.0 M ammonium sulphate gradient yielded two distinct peaks (Figure 1A). When fractions of peaks I and II were detected for inhibition activity, only protein from peak II had apparent antimicrobial activity (Figure 1B), and SDS/PAGE yielded a single band of nearly 32 kDa (Figure 1C). This 32 kDa protein was named AP_1. The results of each purification step are summarized in Table 1.

Inhibition spectrum against pathogens

AP_1 was determined mainly on the basis of its inhibition activity against an indicator bacterial strain PO_{41} of *R. solanacearum in vitro*. Further tests were performed for comparing efficacies against other strains of *R. solanacearum*. The inhibition spectrum investigation showed that AP_1 had good inhibition activity against the five tested strains belonging to *R. solanacearum* from different hosts and geographical locations. The order of inhibition efficacies was $PO_{41} > TM_1 > PO_1 > P_8 > TB_{23}$ and was determined by comparing inhibition zones with 2.5 μg of final protein concentration (Figure 2). The inhibition activities against two fungal pathogens, *R. solani* and *A. solani*, on potato were observed, being localized to regions at the edge of the fungal colony where growth inhibition was evident with 2.5 μg of final protein concentration (Figure 3). The minimum dose of AP_1 protein against pathogens is shown in Table 1.

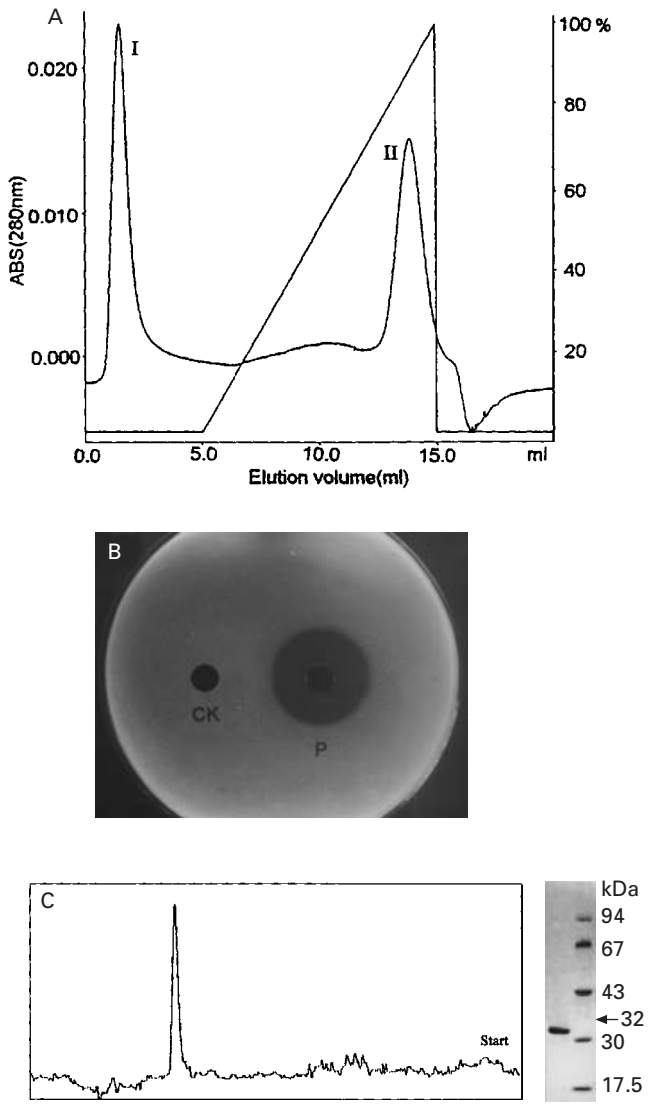

Figure 1 Purification and activity of AP_1 from potato variety MS-42.3 at the hydrophobic Butyl-Sepharose step

(**A**) Elution profile from FPLC purification step with a hydrophobic Butyl-Sepharose column (1 ml). Elution was carried out with 2.0–0.0 M linear gradient of ammonium sulphate at 0.5ml/min. The fraction size of peak I and II was 16 ml. Eluates were measured by their protein content and A_{280}. (**B**) Detection of inhibition activity against *R. solanacearum* PO_{41} strain. The fractions of peaks I and II were pooled separately, dialysed and lyophilized to dry powder. Proteins were dissolved with 1 ml of distilled water. Protein sample (50 μl, 2.5 μg of protein) was injected into 6mm-diameter wells. The plate was incubated at 30 °C and inhibition zones were measured after 48 h. The protein from peak II appeared in the inhibition zone. (**C**) SDS/PAGE analysis of peak II yielding a single band of 32 kDa (right panel). A single peak was seen on the scanning profile (left panel).

Table 1 Purification of AP_1 from potato leaves

Step and fraction	Total protein (mg)*	Minimum dose (μg)	Total activity (nkat)	Specific activity (nkat · mg^{-1})	Purification (fold)	Yield (%)
Soluble protein extraction	10720	34	315	29	1	100
40–70 % ammonium sulphate precipitate fraction (dialysed)	1680	11	150	90	3.1	47.6
Sephacryl S-200	18	1	18	1000	34.5	5.7
Butyl-Sepharose	5.3	0.5†	11.4	2100	72.4	3.6

* Data were referred to 800 g of MS-42.3 leaves of potato.
† The minimum dose for inhibition indicator strain PO_{41} of *R. solanacearum*.

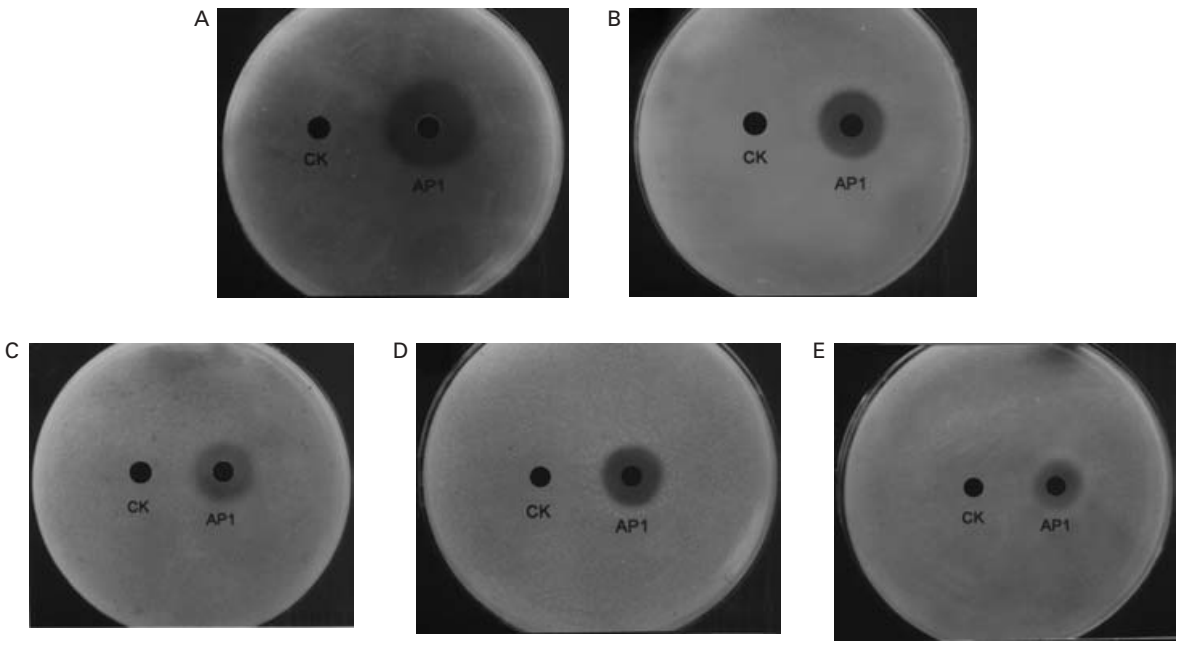

Figure 2 Inhibition effect of AP_1 on different strains of *R. solanacearum*

The inhibition tests of AP_1 against *R. solanacearum*. Different strains were measured in plates by using the agar-well diffusion method. Bacterial suspension (100 μl) of PO_{41}, PO_1, TM_1, TB_{23} and P_8 strains (final concentration 5×10^5 cfu/ml) was mixed in nutrient agar medium. Protein sample (50 μl, 2.5 μg of final protein concentration) was injected into 6 mm-diameter wells. The plates were incubated at 30 °C and inhibition zones were measured after 48 h. (**A**) PO_{41} from potato host; (**B**) TM_1 from tomato; (**C**) PO_1 from potato; (**D**) P_8 from groundnut; (**E**) TB_{23} from tobacco.

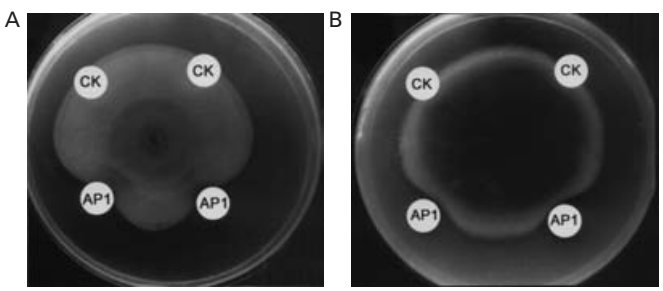

Figure 3 Inhibition effect of AP_1 on fungal pathogens *R. solani* and *A. solani*

The inhibition tests of AP_1 against *R. solani* (**A**) and *A. solani* (**B**) were performed by using agar-well diffusion method. Protein sample (50 μl, 2.5 μg final protein concentration) was injected into 6 mm-diameter wells. The plates were incubated at 18 °C and the inhibition effect was observed after 30–72 h. CK, control.

Isolation of a cDNA clone encoding AP_1

A cDNA expression library of the potato was constructed with mRNA isolated from leaves of the potato (MS-42.3 variety). A library containing 6×10^5 cDNA was obtained. Approx. 3×10^5 recombinants were screened with anti-AP_1 antibody as probe. The positive plaques appeared an intense purple colour at the site of antigen–antibody complexes. The fusion proteins of interest were then isolated from bacteriophage lysogen. The positive clones that might carry target cDNA encoding AP1 were further identified using Western blot analysis with anti-AP_1 antibody and fusion proteins from candidate clones. After *Eco*RI digestion, the target cDNAs were directly subcloned into the pGEM-7Zf(+) plasmid, and sizes of inserts were analysed. Unfortunately, all the positive clones obtained lacked the 5′-end and the size of the inserts was approx. 500 bp. But these inserts showed identity to the C-terminus of AP_1 protein. To obtain the full-length cDNA, a primer was designed based on 3′-end cDNA sequence to carry out RACE PCR using mRNA isolated from MS-42.3 variety leaves as template. A full-length AP_1 cDNA was successfully obtained by screening a cDNA expression library with antibody against AP_1 and RACE-PCR strategies. We have identified a single open reading frame of 1029 bp encoding a predicted AP_1 protein of 343 amino acids. Interestingly, the predicted amino acid sequences apparently possess two different domains. Computer-assisted sequence alignment further revealed

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M. loti  QPSGTDPAPG GDPLLADPAK PTTLPPQTEP TIGDMLSLKH ISWAWYSGAW  340
AP1      MQPSASDTTG K---LADPAA ASTLPPQTGQ TIGDLLSAKG LSWAWYAGSW   47

M. loti  Q-YTLDHG-- NKSPIPNFQY HHQPFNYYAN YAPGTEARRE HLRDGGLGGV  387
AP1      NTASSDRTVI YNNKVPNLQS HHQPFNYYAA FDPVAHPDAR AAHLKDFDS-   96

M. loti  SFIQAIDDGA LPQVSFYKPQ GNVNEHSGYA DIEAGDRHIA DVVAHLEKSP  437
AP1      DFLKDVAAGT LPAVSFYKPQ GNLNQHPGYA SVADGDAHIA DVLAKLQASP  146

M. loti  QWQHMLVVVT YDENGGIWQH VAPPKGDRWG PGTRVPAIIV SPFAKHGYID  487
AP1      QWKNMLVVVT YDENGGFYDH ATVPKGDRWG PGTRIPALIV SPFAKKGFVD  196

M. loti  QTPYDTTSIL RFITERFELP ---------- ---------- ----------  507
AP1      KTQYDTGSTL RFITHRWSLP GAEIFTNNKA TELALPLVEA NVPFKIHIVK  246

         ---------- ---------- ---------- ---------- ----------
AP1      DRDMKERLCL EVERLRLSAM IMGSRGFGAN DIRGIISKGK LGSVSDYCVK  296

         ---------- ---------- ---------- ---------- --------
AP1      NCICPVVVVR YPQEDEYGDG AVEKKRRLRL NCHRYRRRST SIMMPMS     343
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Figure 4 Deduced amino acid sequence encoded by the AP_1 gene and alignment to acid phosphatase from *M. loti*

The protein sequence deduced from the AP_1 cDNA (accession number AY297449) was aligned with closely similar acid phosphatase sequences of *M. loti* (accession number NC002678.1).

that the N-terminus (amino acids 2–217) is related to acid phosphatase found in bacteria [20] (Figure 4), and at the C-terminus (269–306) there is an ATP-binding domain seen in UspA (universal stress protein A) [21,22]. In the ATP-binding domain, the sequence G^{269}SRG-RG–LGSV–CPVXVVR^{306} is a well-conserved domain in UspA, mainly related to nucleotide-binding proteins and signal transduction mechanisms [23]. The N-terminus of AP_1 is most closely related to acid phosphatase from *Mesorhizobium loti* (accession number NC002678.1) and from *Burkholderia pseudomallei* (accession number AF252862.1), and shows 58% and 53% identities respectively. However, AP_1 still shows a weaker relationship with proteins found in plants that have been deposited in GenBank®.

Expression of AP_1 gene in *E. coli* and characterization of the AP_1-fusion protein

In order to obtain definitive evidence that the AP_1 cDNA can be expressed in a prokaryotic system and show anti-bacterial activity, the coding sequence of AP_1 gene was cloned in pET-5a expression vector to produce a fusion protein. An approach was chosen to facilitate the purification method of the fusion protein and to avoid possible contamination with the *E. coli* proteins which might show a similar molecular mass to the AP_1-fusion protein. *E. coli* BL21(DE_3) pLysS was transformed with the empty plasmid pET-5a as a control. Sometimes, a high level of expression of foreign protein in *E. coli* resulted in the formation of inclusion bodies in the pellet. In this case, it was necessary to convert inclusion bodies to soluble protein. Expression of the AP_1-fusion protein was found to depend on the concentration of the transcriptional inducer, IPTG, and was stably overproduced with a fusion partner protein, resulting in the efficient formation of inclusion bodies in *E. coli* BL21(DE3). Most of the AP_1-fusion protein accumulated in insoluble pellets as inclusion bodies (results not shown). The SDS/PAGE pattern of over-expression of the fusion protein induced by IPTG showed that the band corresponding to the 32 kDa AP_1 protein (including 10 amino acids from vector) had a slight increase in molecular mass (Figure 5A, lane II), which was not present in *E. coli* cells transformed

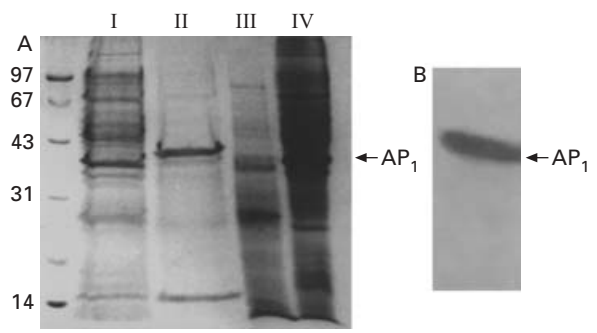

Figure 5 SDS/PAGE and Western blot analysis of AP_1-fusion protein produced in *E. coli*

(**A**) The fusion proteins were run on an SDS/PAGE gel and stained with Coomassie Brilliant Blue. The migration of molecular-mass standards are indicated in kDa on the left-hand side. Lane I, the soluble fractions expressed by pET-5a-AP_1 without IPTG induction. Lane II, the inclusion body fractions of pET-5a-AP_1 with 0.4 mM IPTG induction. The inclusion bodies were dissolved using 100 μl of lysis buffer with 6 M urea and 0.1 mM PMSF. The position of the AP_1-fusion protein is shown by an arrow on the right-hand side. Lanes III and IV, the soluble fractions of pET-5a empty vector transformants. Each sample (15 μl) was loaded into the bottom of the wells and 15 V/cm of voltage was applied. (**B**) Western blot of AP_1-fusion protein in the extract of IPTG-induced pET-5a-AP_1 transformants.

with the expression vector without insert (Figure 5A, lanes III and IV). These results indicated that the high-level of the AP_1-fusion protein had been successfully expressed in *E. coli*.

Western blotting was performed using the fusion protein extracts from *E. coli* BL21(DE3) pLysS expression products and anti-AP_1 antibody. The fusion protein produced by pET-5a-AP_1 was run alongside the empty plasmid pET-5a as a control. The result showed that the anti-AP_1 antibody specifically recognized the fusion protein produced by pET-5a-AP_1, and appeared as a single band in the position corresponding to the molecular mass of AP_1 protein (Figure 5B). None of proteins from empty plasmid pET-5a were recognized by the anti-AP_1 antibody (results not shown). The recognition of the fusion-protein extracts by the anti-AP_1 antibody is strong evidence that AP_1 gene can be successfully

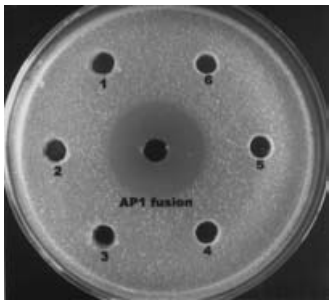

Figure 6 Inhibition activity of AP_1-fusion protein against *R. solanacearum* on plates

Bacterial suspension (100 μl) of *R. solanacearum* PO_{41} strain (final concentration 5×10^5 cfu/ml) was mixed with 20 ml of NA medium. Protein sample (50 μl) was injected into each 6 mm-diameter well. The plate was incubated at 30 °C for 48 h. Well 1, pET-5a-AP_1 (without IPTG induction); wells 2 and 3, empty pET-5a vector (with or without IPTG induction respectively); well 4, sample buffer [50 mM Tris/HCl (pH 7.5), 2 mM EDTA and 0.1 M NaCl]; well 5, 0.01 % (w/v) lysozyme; well 6, distilled water. The central well showed an apparent inhibition zone because of addition of 30 μg of AP_1-fusion protein.

expressed in a prokaryotic system and had the ability to form antigen–antibody complexes.

Antibacterial property of AP_1-fusion protein

In order to detect whether or not the AP_1-fusion protein expressed in *E. coli* still kept its characteristic of antibacterial activity, inhibition tests of bacterium growth were conducted in plates. Apparent inhibition zones were observed around the wells with AP_1-fusion protein (induced by IPTG), but none of other samples, with proteins expressed by pET-5a-AP_1 uninduced by IPTG, empty pET-5a vector (induced by IPTG or not), sample buffer [50 mM Tris/HCl (pH 7.5), 2 mM EDTA and 0.1 M NaCl] and 0.01 % (w/v) lysozyme showed inhibition zones (Figure 6). These results indicated that an AP_1-fusion protein over-expressed in *E. coli* exhibited its inhibition activity.

DISCUSSION

We successfully purified an antimicrobial protein, AP_1, from the bacterial wilt-resistant germ plasm from potato. This protein is a homologue of an acid phosphatase from *M. loti*. Interestingly, AP_1 shares two different domains, acid phosphatase at the N- terminus and an ATP-binding domain at the C-terminus, which is mainly related to nucleotide-binding proteins and signal transduction mechanisms [23]. AP_1 shows a weaker relationship (10–15 % identity) with acid phosphatases reported from plants (accession numbers: AF200824, soya bean; AF200825, sweet potato; AC003076, *Arabidopsis thaliana*). In potato, an acid phosphatase StPPP1 (*S. tuberosum* phosphate starvation-induced phosphatase) isolated by cDNA amplified fragment length polymorphism in response to bacteria, has high homology to a phosphate starvation-induced acid phosphatase from tomato (*LePS2*). Decreases in phosphate availability after pathogen infection act as a signal for the activation of the potato phosphatase gene [24,25], but it shares only 5 % identity with AP_1.

It was reported that most proteins with antimicrobial activity *in vitro* belong to a group of small molecular mass antimicrobial peptides from plants [4,5], and were rarely linked to the acid phosphatase family. We have isolated and purified AP_1, mainly on the basis of its antimicrobial activity *in vitro*, but the function of AP_1 is still not clear. Now, we justifiably suggest that AP_1 belongs to a new protein group with characteristics of an antimicrobial. Further investigations on the mechanisms of AP_1 against potato pathogens at the molecular level will greatly enhance our understanding of its roles in plant disease resistance or signal transduction.

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REFERENCES

1 Karin, T., Rupert, W. O., David, P. A. and Willem, F. B. (2000) Specific binding sites for an antifungal plant defensin from Dahlia (*Dahlia merckii*) on fungal cells are required for antifungal activity. Mol. Plant – Microbe Interact. **13**, 54–61
2 Scheel, D. (1998) Resistance response physiology and signal transduction. Curr. Opin. Plant Biol. **1**, 305–310
3 Fritig, B., Heitz, T. and Legrand, M. (1998) Antimicrobial proteins in induced plant defense. Curr. Opin Immunol. **10**, 16–22
4 García-Olmedo, F., Rodriguez-Palenzuela, P., Molina, A., Alamillo, J. M., Lopez-Solanilla, E., Berrocal-Lobo, M. and Poza-Carrion, C. (2001) Antibiotic activities of peptides, hydrogen peroxide and peroxynitrite in plant defense. FEBS Lett. **498**, 219–222
5 García-Olmedo, F., Molina, A., Alamillo, J. M. and Rodríguez-Palenzuela, P. (1998) Plant defense peptides. Biopolymers **47**, 479–491
6 Broekaert, W. F. , Cammue, B. P. A., De Bolle, M. F. C., Thevissen, K., De Samblanx, G. W. and Osborn, R. W. (1997) Antimicrobial peptides from plants. Crit. Rev. Plant Sci. **16**, 297–323
7 García-Olmedo, F., Carmona, M. J., Lopez-Fando, J. J., Fernandez, J. A., Castagnaro, A., Molina, A., Hernandez-Lucas, C. and Carbonero, P. (1992) Characterization and analysis of thionin genes. In Genes Involved in Plant Defence (Boller, T. and Meins, F., eds.). pp, 283–302, Springer-Verlag, Vienna
8 García-Olmedo, F., Molina, A., Segura, A. and Moreno, M. (1995) The defensive role of nonspecific lipid-transfer proteins in plants. Trends Microbiol. **3**, 72–74
8a Yabuuchi, E., Kosako, Y., Yanom, I., Hotta, H. and Nishiuchi, Y. (1995) Transfer of two *Burkholderia* and an *Alcaligenes* species to *Ralstonia gen*. Nov.: Proposal of *Ralstonia pickettii* (Ralston, Palleroni and Doudoroff 1973) comb. Nov., *Ralstonia solanacearum* (Smith 1896) comb. Nov. and *Ralstonia eutropha* (Davis 1969) comb. Nov. Microbiol. Immunol. **39**, 897–904
9 Hammond-Kosack, K. E., Tang, S., Harrison, K. and Jones, J. D. (1998) The tomato Cf-9 disease resistance gene functions in tobacco and potato to confer responsiveness to the fungal avirulence gene product avr 9. Plant Cell **10**, 1251–1266
10 Kawchuk, L. M., Hachey, J., Lynch, D. R., Kulcsar, F., van Rooijen, G., Waterer, D. R., Robertson, A., Kokko, E., Byers, R., Howard, R. J. et al. (2001) Tomato Ve disease resistance genes encode cell surface-like receptor. Proc. Natl. Acad. Sci. U.S.A. **98**, 6511–6515
11 Lorito, M., Woo, S. L., Garcia, I., Colucci, G., Harman, G. E., Pintor-Toro, J. A., Filippone, E., Muccifora, S., Lawrence, C. B., Zoina, A. et al. (1998) Genes from mycoparasitic fungi as a source for improving plant resistance to fungal pathogens. Proc. Natl. Acad. Sci. U.S.A. **95**, 7860–7865
12 Gao, A. G., Hakimi, S. M., Mittanck, C. A., Wu, Y., Woerner, B. M., Stark, D. M., Shah, D. M., Liang, J. and Rommens, C. M. (2000) Fungal pathogen protection in potato by expression of a plant defensin peptide. Nat. Biotechnol. **18**, 1307–1310
13 Wu, G., Shortt, B. J., Lawrence, E. B., Fitzsimmons, K. C. and Shah, D. M. (1995) Disease resistance conferred by expression of a gene encoding H_2O_2-generating glucose oxidase in transgenic potato plants. Plant Cell **7**, 1357–1368
14 Jia, S. R., Qu, X. M., Feng, L. X. and Tang, X. (1996) Progress in the genetic engineering of potato cultivars for resistance to bacterial wilt in 1987–1995. In Genetic Engineering of Potato by Introducing Antimicrobial Peptides (Jia, S.R. and Qu, X.M., eds.), pp. 1–12, The China Agricultural Science and Technology Press, Beijing
15 Berrocal-Lobo, M., Segura, A., Moreno, M., Lopez, G., Garcia-Olmedo, F. and Molina, A. (2002) Snakin-2, an antimicrobial peptide from potato whose gene is locally induced by wounding and responds to pathogen infection. Plant Physiol. **128**, 951–961
16 Gebhardt, C. and Valkonen, J. P. (2001) Organization of genes controlling disease resistance in the potato genome. Annu. Rev. Phytopathol. **39**, 79–102
17 He, L. Y., Sequeira, L. and Kelman, A. (1983) Characteristics of strains of *Pseudomonas solanacearum* from China. Plant Dis. **12**, 1357–1361
18 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

19 Smith, B. J. (1984) SDS polyacrylamide gel electrophoresis of proteins. Methods Mol. Biol. **1**, 41–55
20 Kaneko, T., Nakamura, Y., Sato, S., Asamizu, E., Kato, T., Sasamoto, S., Watanabe, A., Idesawa,K., Ishikawa, A., Kawashima, K. et al, (2000) Complete genome structure of the nitrogen-fixing symbiotic bacterium *Mesorhizobium loti*. DNA Res. **31**, 331–338
21 Mushegian, A. R. and Koonin, E. V. (1996) Sequence analysis of eukaryotic developmental proteins: ancient and novel domains. Genetics **144**, 817–828
22 Nystrom, T. and Neidhardt, F. C. (1994) Expression and role of the universal stress protein, UspA, of *Escherichia coli* during growth arrest. Mol. Microbiol. **11**, 537–544
23 Zarembinski, T. I., Hung, L. W., Mueller-Dieckmann, H. J., Kim, K. K., Yokota, H., Kim, R. and Kim, S. H. (1998) Structure-based assignment of the biochemical function of a hypothetical protein: a test case of structural genomics. Proc. Natl. Acad. Sci. U.S.A. **95**, 15189–15193
24 Baldwin, J. C., Karthikeyan, A. S. and Raghothama, K. G. (2001) LePS2, a phosphorus starvation-induced novel acid phosphatase from tomato. Plant Physiol. **125**, 728–737
25 Petters, J., Gobel, C., Scheel, D. and Rosahl, S. (2002) A pathogen-responsive cDNA from potato encodes a protein with homology to a phosphate starvation-induced phosphatase. Plant Cell Physiol. **43**, 1049–1053

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