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Characterization of a Phytoplasma-related Ribonuclease in *Catharanthus roseus* (L.) G. Don

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ABSTRACT : A 6.8 kDa ribonuclease protein was found in the protein extract of the diseased periwinkle (*Catharanthus roseus* (L.) G. Don) infected by the phytoplasma of loofah witches' broom, but not in that of the healthy plants. This protein has an isoelectric point between 4 and 5. Further analyses revealed that it had a broad optimum pH of 3 to 8, and was sensitive to inhibition by $CaCl_2$ (2 mM) and $MgCl_2$ (2 mM) and less seneitive to $ZnCl_2$ (50 μM), but insensitive to EDTA (250 mM) and NaCl (5mM).

In order to investigate if this RNase could also be induced by other factors, the healthy periwinkle were given different environmental stress treatments including heat shock, drought, ethanol, and abscisic acid. None of these treatments induced the expression of this phytoplasma-related RNase protein.

KEY WORDS: Loofah witches' broom, Phytoplasma, Ribonuclease.

INTRODUCTION

Phytoplasmas are a group of pleomorphic, wall-less bacteria with a diameter of 80-800 nm and were first found in the phloem elements of several diseased plants including mulberry dwarf, potato witches' broom, paulownia witches' broom and aster yellows by Doi *et al.* (1967). Prior to 1993, they are named as mycoplasma-like organisms (MLOs) because their resemblance to animal mycoplasma in morphology and ultrastructure (Bove *et al.*, 1989, for review). The following molecular evidences support that MLOs are members of the class Mollicutes: (1) The genome complexity is within the range of many mycoplasma species, from 450 to 1180 kb; (2) The genomic G+C content of several MLOs is 25 to 30 mol%, characteristic of other members of the Mollicutes. However, comparisons of the DNA sequence of 16S rRNA and ribosomal protein genes of several mollicutes show that MLOs are more closer phylogenetically to *Acholeplasma* species, *Anaeroplasma* species and *Asteroleplasma* species than to *Spiroplasma* species and *Mycoplasma* species (Sears and Kirpatrick, 1994). Thus, the Subcommittee on the Taxonomy of Mollicutes of the International Committee on Systematic Bacteriology agreed a proposal to replace the term MLOs with phytoplasmas (Tully, 1993).

Phytoplsmas have received a wide interest for several reasons. In addition to their small genome, phytoplasmas are found in association with many serious plant diseases,

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damaging important crops and resulting in a severe worldwide economic loss (McCoy *et al.*, 1989). However, the inability thus far to culture and isolate these organisms *in vitro* has limited the study of phytoplasmas.

The investigation described here was designed to study the possible role of ribonuclease (RNase) of phytoplasma of loofah witches' broom in pathogenesis. Ribonuclease and deoxyribonuclease have been demonstrated as the only two enzyme activities found in the peripheral membrane protein fraction of several mollicutes (Razin, 1982). The nuclease activities in the *Mycoplasma pulmonis* membrane was also observed and was proposed to serve a vital function in the pathogenesis of this organism (Minion and Goguen, 1986). Furthermore, addition of adenine could spare the pathological effects on hamster tracheal ring cultures infected with *Mycoplasma pneumoniae* (Gabridge and Stahl, 1978).

In this paper, we report a ribonuclease, named phytoplasma-related ribonuclease, found in the protein extract of periwinkle infected by the phytoplasma of loofah witches' broom, and its biochemical properties.

MATERIALS AND METHODS

Plant material

Phytoplasmas of loofah witches' broom were maintained by graft inoculation in periwinkle (*Catharanthus roseus* (L.) G. Don). Diseased periwinkle was originally provided by Dr. H.-J. Su, Department of Plant Pathology and Entomology, National Taiwan University.

Preparation of protein extracts

Small diseased or healthy periwinkle branches were frozen in liquid nitrogen, and stored in -80℃ freezer. The frozen tissues were ground with a cold mortar and pestle. The protein extract was carried out at 4℃ as previously described (Liou et al., 1995) by adding 3.5 ml/g tissue of extraction buffer (20 mM Tris-HCl, pH 7.4, 0.01M EDTA, 20 mM DTT, 0.05 M KCl, 5 % glycerol) to the ground tissue, and the sample was continually pestled. The homogenate was centrifuged at 12,000 g for 15 min. The supernatant was used for the enzyme assay. The supernatants were stored at -20 ℃ until the assay was performed.

Protein concentration in the supernatant was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Biochemical and enzymatic assays

The RNase activity was determined by the substrate-base gel assay according to the method described by Blank *et al.* (1982). Briefly, the protein extracts were electrophoresed on a 15% RNA-cast SDS-PAGE. After electrophoresis, the SDS was removed from the gel by washing the gel with 25 % isopropanol, and the proteins were renatured in 10 mM Tris-HCl, pH 7.4. The RNase activities bands were demonstrated by incubating the gel at 55 ℃ for two hours in 10 mM Tris-HCl, pH 7.4, followed by toluidine blue O (0.2%) staining and destaining in 10 mM Tris-HCl, pH 7.4 at room temperature. The RNase activity were shown as the colorless band against a blue background.

For studying the effects of different factors on the RNase activity, the chemicals were added to the pre-incubation or incubation buffer before gel electrophoresis or to gel incubation solution at 55 ℃ after electrophoresis.

Two-dimensional gel electrophoresis

The first dimension gel analysis was performed as described by O' Farrell (1975) and carried out in a Hoeffer GT apparatus. The proteins were solubilized in the solution containing 9.5 M urea, 0.2 % NP-40, 5 % β-mercaptoethanol and 5% ampholine (pH 3.5 to 10, Pharmacia). 100 μg of protein were loaded at the upper acid end of the focusing gel. Electrode solutions were 0.5 % ethanoamine at the cathod and 0.2 % H_2SO_4 at the anode. The isoelectric focusinng was done for 17 hours at 250 volts and 1 hours at 500 volts. Gels were then equilibrated in the solution countaining 10 % glycerol, 23 % SDS, 5 % β-mercaptoethanol and 0.062 M Tris-HCl, pH 6.8 for 45 min. The second dimensional electrophoresis was done on a substrate-base assay gel.

The effects of envirnmental stresses on the expression of phytoplasma-related RNase

In order to determine the appearance of the extra RNase in the diseased plant is related to the phytoplasma infection, the healthy periwinkle or its leaves were treated with different environmental stresses as previously described, then proteins were extracted from the leaves for the RNase activity assay: (A) Heat shock treatment (Tzeng *et al.*, 1993): The leaves were immersed in different flasks countaining the shaking solution (1 mM potassium phosphate, pH 6.0, 1 % sucrose). The flask was incubated in the water bath of 28 ℃, 31 ℃, 34 ℃, 40 ℃ or 42 ℃ for 2 hours. (B) Drought treatment (Kahn *et al.*, 1993): The plant was at drought condition for 2 days. (C) Ethanol treatment (Sanchez *et al.*, 1992): The leaves were immersed in the shaking solution containing 5 % or 10 % ethanol for 2 hours. (D) Abscisic acid treatment: The peteoles of leaves were immersed in water containing 0.01, 0.1 or 1 mM abscisic acid for 6 hours.

RESULTS

Syndrome of the periwinkle infected by the phytoplasma of the loofah witches' broom

The successful graft of a diseased branch on the plant hosts resulted in the production of phyllody and witches' broom or thinner stems with little leaves (Fig. 1) as described previously (Yang *et al.*, 1974).

The RNase profiles of healthy and diseased plants

The crude protein extracts of healthy and diseased periwinkle were subjected to substrate-base gel electrophoretic analyses. There were two major groups of RNases bands with a molecular weight of 21.5 kDa and 26.7 kDa, respectively, in the protein extracts of both healthy and diseased plants (Fig. 2). However, there was an extra RNase of 6.8 kDa, named as phytoplasma-related RNase in diseased plant protein extract. This extra RNase was associated with the phytoplasma infection, because it was not present in the protein extracts of other periwinkle strain and the senescent leaves. Futhermore, the expression of this extra RNase could not be induced by several environmental stress treatments (Fig. 3).

Fig. 1. Syndrome of the periwinkle infected by the phytoplasma of the loofah witches' broom : (A) a branch of healthy periwinkle. (B) a branch of diseased periwinkle. White bar at the bottom equals 5 cm.

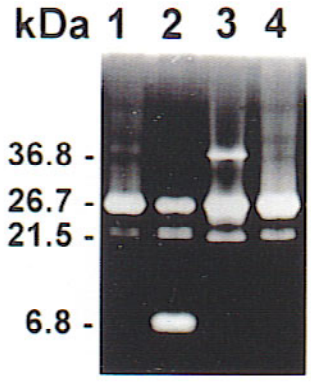

Fig. 2. The RNase profiles of healthy and diseased plants. Each well was loaded with 15 μg of protein extract. Lane 1, healthy periwinkle; lane 2, diseased periwinkle; lane 3, senescent periwinkle leaves; lane 4, short-stem periwinkle.

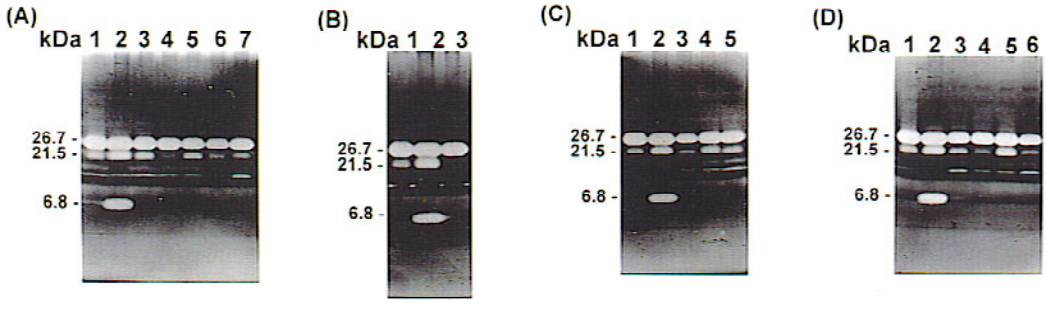

Fig. 3. The effect of environmental stress on the expression of RNase. Each well was loaded with 15 μg of protein extract. Lane 1 and lane 2 in each panel are healthy and diseased plant, respectively. (A) Heat shock treatment: 28 ˚C (lane 3), 31˚C (lane 4), 34˚C (lane 5), 40˚C (lane 6) and 42 ˚C (lane 7). (B) Drought treatment: Lane 3, drought plant. (C) Ethanol treatment: shaking solution only (lane 3), 5 % ethanol (lane 4) and 10% ethanol (lane 5). (D) Abscisic acid treatment: water only (lane 3), 0.01 mM abscisic acid (lane 4), 0.1 mM abscisic acid (lane 5) and 1 mM abscisic acid (lane 6).

Fig 4. shows the two-dimensional gel analyses for the protein extract from healthy and diseased plants. Interestingly, all the three groups of RNases have the same isoelectric point between 4 and 5.

Biochemical analysis of phytoplasma-related RNase properties

The effects of pH, cations, EDTA, and β-mercaptoethanol on the RNase activity were investigated by adding the chemicals to the pre-incubation or incubation buffer before the samples were loaded to the gel or to the gel incubation solution at 55 ˚C after electrophoresis. In all experiments, the RNases of 21.5 kDa and 26.7 kDa were used for the comparison. Table 1 showed that in comparing with other two RNases, the phytoplasma-related RNase was more sensitive to the inhibition by $CaCl_2$ and $MgCl_2$, but less sensitive to $ZnCl_2$ (Fig. 5). Phytoplasma-related RNase was insensitive to NaCl and EDTA. β-mercaptoethanol displayed an inhibitory effect on all of these groups of RNases at a concentration of 5 %. All three groups of RNases had a relatively broad range of pH (fig. 6).

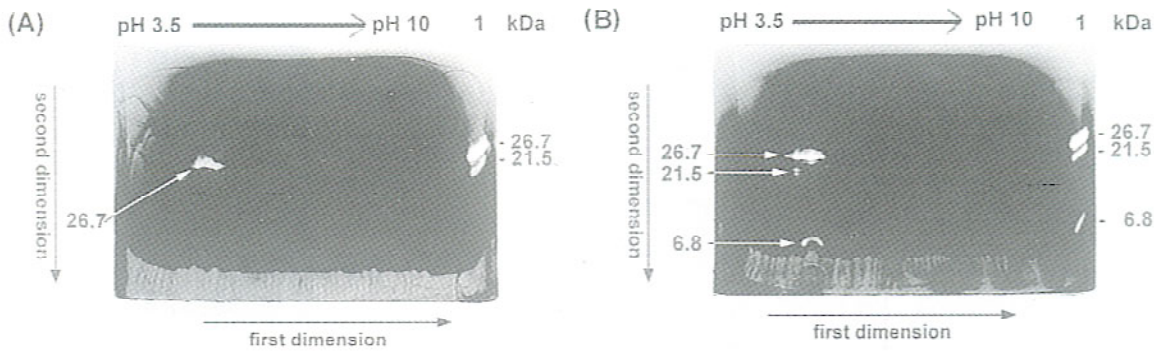

Fig. 4. Two-dimensional gel analyses of (A) diseased and (B) healthy plant protein extracts. Lane 1 is an one-dimensional electrophoresis of the protein extract. In (B) the RNase of 21.5 kDa is someway missed. The arrows point out the increasing directions of the pH and the directions of electrophoresis as indicated.

Table 1: The RNase properties of healthy and diseased periwinkles.

	major group	minor group	phytoplasma-related RNase
M. W.	26.7 KDa	21.5 KDa	6.8 KDa
Ca^{2+}	N	N	2 mM^{+}
Mg^{2+}	N	N	2 mM
Zn^{2+}	5 μM*	5 μM**	50 μM
K^{+}	500 mM	100 mM	500 mM
Na^{+}	250 mM	50 mM*	N
pH range	4-9	3-8	3-8
EDTA	enhance (2 mM)	5 mM	N
β-mecaptoethanol	5%	5%	5%

$^{+}$ The concentration of chemical shown indicates that an initial inhibition was exhibited.
* A complete inhibition.
** A difference in the inhibition to the 21.5 KDa RNases of healthy and diseased plants was shown (also see Fig. 5).
N No effect.

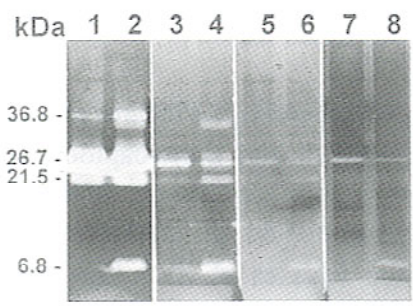

Fig. 5. The effect of $ZnCl_2$ on the RNase activity. Lanes with odd number were loaded with the protein extract from healthy plants, and lanes with even number were from the diseased plants. Each lane was loaded with 15 μg of protein extract. The different concentration of $ZnCl_2$ was added to the gel incubation buffer. Lanes 1, 2,: no $ZnCl_2$ addition. Lanes 3, 4: 5 μM $ZnCl_2$. Lanes 5, 6: 50 μM $ZnCl_2$. Lanes 7, 8: 200 μM $ZnCl_2$.

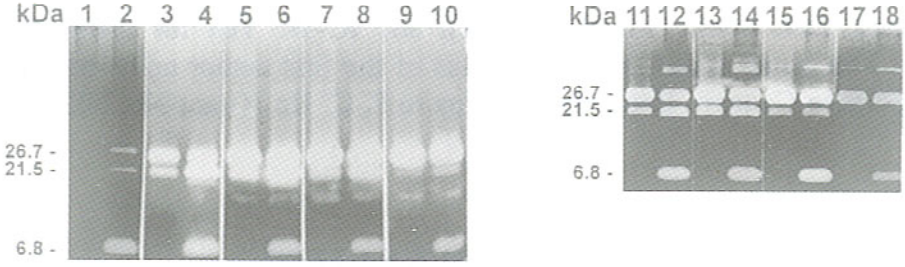

Fig. 6. The pH range of RNase activity. The lanes with odd number were loaded with the protein extract from the healthy plants, and the lane with even number were from the diseased plants. Each lane was loaded with 15 μg of protein extract. The gels were immersed in different pH gel incubation buffer. Lane 1 to 10 were in citrate / Na_2HPO_4 buffer, and lane 11 to 18 were in Tris-HCl buffer. Lanes 1, 2: pH 3; lanes 3, 4: pH 4; lanes 5, 6: pH 5; lanes 7, 8: pH 6; lanes 9, 10: pH 7; lanes 11, 12: pH 7; lanes 13, 14: pH 7.4; lanes 15, 16: pH 8; lanes 17, 18: pH 9.

DISCUSSION

RNases of higher plants have been grouped into four main families based on pH optimum, molecular mass and sensitivity to EDTA. These RNases have a molecular weight between 20 kDa and 100 kDa with a pH optimum of 5 to 7 and 7 to 9 (Yen and Green, 1991). Apparently, the phytoplasma-related RNase does not belong to these categories.

Low molecular RNases have been identified previously. A 9 kDa RNase was reported to appear periodically in the protein extract of *Arabidopsis thaliana* (Yen and Green, 1991). This RNase has an optimal pH 6.5, and its activity is enhanced by $ZnCl_2$. Liou *et al.* (1995) also reported a 6 kDa RNase (group F) in the leaves of senescent or dark-growth sweet potato seedlings. In this study, the phytoplasma-related RNase is not stress-associated, and exhibited a similar activity from pH 3 to 8 (Table 1 and Fig. 6). EDTA and $ZnCl_2$ had little effect on its activity (Table 1 and Fig. 5). These data indicate that this disease-induced RNase belongs to different category.

RNases are classically defined as the enzymes that cleave RNA specifically. However, recent data show that RNases have been active in many biological actions including angiogenesis, antitumor, antifertility, immunosuppression, neurotoxicity, pollen control, starvation rescue, fruit development, host defense and even function as antibiotics (D'Alessio *et al.*, 1991; D'Alessio, 1993). D'Alessio suggested to use the acronym RISBASEs (Ribonucleases with Special, i.e. non-catalytic, Biological Actions) for these RNases with special biological actions. The RNase interests us as they might play a role in the pathogenesis of phytoplasma.

Our experiments clearly demonstrate that a phytoplasma-related RNase is associated with the infection of periwinkle by the phytoplasma of loofah witches' broom. This RNase does not exist in the healthy periwinkles stressed by different factors. However, we can not determine at this time whether this enzyme is encoded by phytoplasma genome or induced by the phytoplasma infection until the gene is cloned.

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植物菌質體核醣核酸水解酵素之特性分析

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摘　　要

絲瓜簇葉病之病原，植物菌質體 (phytoplasma) 感染日日春，自病株抽取蛋白質，做核醣核酸水解酵素活性分析發現有一酵素蛋白質存在於病株，而不存於健康植株的蛋白質抽出液。此蛋白質的分子量約為 6.8 kDa，其等電點介於 4-5 之間。對此酵素性質作進一步的分析，發現它的活性具有寬廣的 pH 範圍，由 3 至 8。且其活性對 $CaCl_2$ 及 $MgCl_2$ 敏感，對 $ZnCl_2$ 較不敏感，但不會受 EDTA 及 NaCl 之抑制。

為了要確定這個水解酵素的出現並非其它環境壓力因子所誘發，因此將健康日日春植物做各種不同的處理，包括加溫、乾旱、酒精、及離層酸等，結果植物體均不能像植物菌質體之感染一樣有 6.8 kDa 的核醣核酸水解酵素的產生。

關鍵詞：絲瓜簇葉病，植物菌質體，核醣核酸水解酵素。

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