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Biochemical and Biophysical Research Communications 312 (2003) 761–766
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cDNA cloning, expression, and mutagenesis of a PR-10 protein SPE-16 from the seeds of *Pachyrrhizus erosus*

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Received 12 October 2003

Abstract

SPE-16 is a new 16 kDa protein that has been purified from the seeds of *Pachyrrhizus erosus.* It's N-terminal amino acid sequence shows significant sequence homology to pathogenesis-related class 10 proteins. cDNA encoding 150 amino acids was cloned by RT-PCR and the gene sequence proved SPE-16 to be a new member of PR-10 family. The cDNA was cloned into pET15b plasmid and expressed in *Escherichia coli*. The bacterially expressed SPE-16 also demonstrated ribonuclease-like activity in vitro. Site-directed mutation of three conserved amino acids E95A, E147A, Y150A, and a P-loop truncated form were constructed and their different effects on ribonuclease activities were observed. SPE-16 is also able to bind the fluorescent probe 8-anilino-1-naphthalenesulfonate (ANS) in the native state. The ANS anion is a much-utilized "hydrophobic probe" for proteins. This binding activity indicated another biological function of SPE-16.
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Keywords: Pathogenesis-related; Ribonuclease; Mutagenesis; ANS-binding activity

The invasion of pathogens, such as fungi, bacteria, and viruses, to plants could normally induce the expression of a series of so called "pathogenesis-related proteins" (PRs). The induction of PRs has been identified in many plant species, suggesting a general role for all these proteins in plant's defensive systems [1]. Since the first "pathogenesis-related" protein was characterized in tobacco leaves induced by tobacco mosaic virus (TMV) [2,3], many other PR proteins have been recognized as chitinases, glucanases, proteinase inhibitors, endoproteinase, and peroxidases in different plant species. Currently, PR proteins were identified and classified into 14 families based on their amino acid sequence, serological relationship, and enzymatic activities [1]. These PR proteins exist widely in plant kingdom from monocot to dicot plant species.

PR-10 family members, which were found in plants such as parsley [6], pea [7], bean [8], potato [9], lupin [10], and lily [11], have been characterized to be small acidic intracellular proteins resistant to proteases [4,5]. Most of them are induced by various stimuli: pathogen, darkness, phytohormones, etc. Besides the induction by stimulus, PR-10 proteins can also be constitutively expressed in various plants, including yellow lupine [12], *Pisum sativum* [13], soy been [14], birch [15], celery [16], and apple [17]. This indicates PR10 proteins could play important roles both in plant defense and normal development. Although the biological function of PR-10 proteins is little known yet, the sequence homology between a ginseng ribonuclease and PR-10 proteins suggested a ribonuclease activity for PR-10 family [18]. However, only a few PR-10 proteins have been reported to demonstrate ribonuclease activity in vitro: Betv 1 [19], LaPR-10 [10], GaPR-10 [20], and LlPR10.1B [23]. There is no homology found between these PR-10 ribonuclease-like proteins with any other plant ribonuclease, but a phosphate-binding loop motif (GXGGXGXXK) shows a remarkably conservation among PR-10 homologous proteins. This motif is also called P-loop,

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0006-291X/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2003.10.181

which was found in nucleotide-binding proteins engaged in ATP or GTP binding [21].

According to the three-dimensional structure of the major birch pollen allergen Bet v1 [22] and two homologous PR10 proteins from yellow lupine [23], PR-10 proteins contain a long C-terminal α-helix wrapped around by a seven-strand β-sheet and two additional N-terminal short α-helices. Three conserved amino acids in Bet v1, Glu-96, Glu-148, and Tyr-150 were predicted to be involved in its ribonuclease activity [24].

In our previous work, we purified and characterized a new PR-10 protein SPE-16 from the seeds of *Pachyrrhizus erosus* [25]. Here we report the cDNA cloning of SPE-16 by RT-PCR and its expression in *Escherichia coli*. In order to elaborate the mechanism of its ribonuclease-like activity, site-directed mutation of three conserved amino acids E95A, E147A, Y150A, and a P-loop truncated form was constructed and showed their different effects on the ribonuclease activity.

A recent report demonstrated that Bet v1 in its native state could bind a variety of both synthetic and naturally small compounds including fluorescent probe ANS (8-anilino-1-naphthalenesulfonate), fatty acids, flavonoids, and cytokinins, which suggested a transport or storage function of Bet v1 [26]. That revealed the ligand-binding activity is important for the biological function of PR10 proteins.

Materials and methods

Total RNA extraction and RT-PCR. Protein purification and N-terminal amino acid sequence have been described before [25]. To get total RNA, the seeds of the *P. erosus* were germinated at 28 °C. After 3 days the germules were collected and homogenated in liquid nitrogen. Total RNA was isolated with Trizol reagent (BioBasic, Canada).

The first strain of cDNA was synthesized with oligo(dT) primer and M-MLV reverse transcriptase. cDNA synthesis was performed at 75 °C for 5 min and followed by 37 °C incubation for 1 h. PCR was carried out with two degenerate oligo-nucleotide primers. The 5′ primer was designed according to the known N-terminal amino acid sequence (GVFVFRDET):5′-GG(A/G/C/T)GT(A/G/C/T)TT(C/T)GT(A/G/C/T)TT(C/T)(T/A)(C/T)(A/G/C/T)GA(C/T)GA(A/G)AC-3′. The 3′ primer was synthesized based on the C-terminal conserved region of the PR-10 family (FKAVETYL): 5′-(A/G/C/T)AG(A/G)TA(A/G/C/T)(G/C)(C/T)(C/T)TCIA(C/T)(A/G/C/T)GC(C/T)TT(A/G)AA(A/G/C/T)A-3′. The amplified 450 bp fragment was purified from 1% agarose gel, ligated into pMD 18-T vector, and sequenced.

Expression and purification. For expressing this gene, two specific primers were synthesized, Primer1 5′-GGAATTCCATATGGGG GTGTTTGTTGTTTTTAG-3′, with an *Nde*I site (underlined). Primer2 5′-GCGCTCGAGTTATAGGTAGGTCTCGATAGC-3′ with an *Xho*I site (underlined). These were used for PCR amplification at an annealing temperature of 55 °C for 30 cycles using sequenced pMD 18-T vector which contained the spe-16 gene. After digestion with *Nde*I and *Xho*I, the PCR products were ligated into the plasmid pET15b and then transformed into *E. coli* BL21(DE3) for protein expression.

BL21(DE3) cell strains transformed with pET15b harboring spe-16 gene were grown in LB (Luria–Bertani) medium containing ampicillin (100 μg/ml) at 37 °C to an absorbance of 0.5–1.0 at 600 nm. The protein expression was induced by the addition of 1 mM IPTG. The cells were harvested three hours after induction and centrifuged (10 min, 5000 rpm, 4 °C). The pellets were resolved in 50 mM Tris–HCl, pH 7.5, buffer containing 0.5 M NaCl, and 1 mM PMSF and lysed by sonication. Following centrifugation at 15,000 rpm, 4 °C for 30 min, the supernatant was loaded onto a Ni-chelating Sepharose Fast Flow gel column (Amersham–Pharmacia Biotech) and washed with a gradient of 100 mM to 1 M imidazole.

Site-directed mutation and P-loop deletion of SPE-16. In Bet v1, Glu-96, Glu-148, and Tyr-150 (Glu-95, Glu-147, and Tyr-149 in SPE-16) were predicted to be involved in ribonuclease activity [24]. Four pairs of primers were designed to prepare the mutation of these three sites and a P-loop truncated form: E95A (5′-GTTGCCTGA AAGTTTG**GCG**AAACTTTCATTCGAG-3′; 5′-CCTGTTCCTCCT ACTATG-3′), E147A (5′-ATTCTTCAAAGCTATC**GCG**ACCTACC TATAACTCG-3′; 5′-CCAGCTCCCTTGGCCAGG-3′), Y149A (5′-CAAAGCTATCGAGACC**GCG**CTATAACTCGAGGATC-3′; 5′-AA GAATCCAGCTCCCTTG-3′), and P-loop (GNGGVGTIK) deletion (5′-AAAATAACCGCTAATGAAGG-3′; 5′-TTCACCGAGTTCAA TACTTTGG-3′). Using pET-15b vector containing spe-16 gene as template, these mutation and deletion were performed by Takara mutanBEST kit. Then they were sequenced and transformed into *E. coli*. Their expression and purification were described above.

1-GGGGTGTTTGTTTTTAGAGACGAGACCTCCTCTAGCGTGGCTCCGGCTAAACTCTAC

AAAGCTCTTACAAAAGATTCCGATACCATCGCCCAAAAGATTGATGGGCCCATCCAAA

GTATTGAACTCGTTGAAGGAAATGGAGGGGTCGGAACCATCAAGAAAATAACCGCTAA

TGAAGGTGACAAAACCAGCTTCGTGCTGCAAAAAGTTGATGCAATCGACGAGGCTAA

CTTGGGATACGACTACAGCATAGTAGGAGGAACAGGGTTGCCTGAAAGTTTGGAGAA

ACTTTCATTCGAGACAAAGGTGGTGGCTGGCTCTGGTGGTGGCTCCATCTCCAAGGTC

ACTCTTAAATTCCACACCAAAGGCGATGCACCCCTTTCAGATGCAGTGCGTGATGATGC

CCTGGCCAAGGGAGCTGGATTCTTCAAAGCTATCGAGACCTACCTA-450

Fig. 1. cDNA sequence of SPE-16.

RNA degradation activity assay. RNase activity of the purified native, recombinant SPE-16 and mutants was carried out at room temperature. The reaction mixtures contained 6 μg total RNA of *P. erosus* and 4 μg proteins in 10 mM imidazole, 5 mM NaCl, and 10 mM Tris–HCl (pH 7.5) buffer. After 1-h incubation, the proteins were removed from the reaction mixtures by extraction with phenol–chloroform. The control mixture described above without SPE-16 protein was also incubated for 1 h at room temperature. The experimental results were observed on 1.2% agarose gel.

ANS-binding activity. Fluorescence experiments were performed on AMINCO. Bowman series 2 Luminescence Spectrometer (Thermo Spectronic, USA). Fluorescence of ANS was excited at 350 nm and emission spectra were recorded between 400 and 600 nm.The concentration of native SPE-16 and ANS in the assay was 1 μM, respectively, in 50 mM different pH buffers. The buffers used as a function of pH were as follows: pH 2.0–6.0, sodium citrate; pH 7.0–8.0, Tris–HCl; and pH 9.0, Bincine. ANS emission spectra in different buffers were subtracted from the corresponding ANS/protein spectra.

Results

Cloning and expression of SPE-16

Two degenerate oligo-nucleotide primers were designed based on the N-terminal amino acid sequence (GVFVFRDET) and the C-terminal conserved sequence (FKAVETYL) of the PR-10 family. A 450 bp fragment from RT-PCR was sequenced (Fig. 1). It encodes a 150 amino acid PR-10 class protein with a predicted molecular mass of 15,676 Da and isoelectric point of 5.02.

The mutation and truncation were proved by DNA sequencing. Recombinant SPE16 and mutations purified by chelating Sepharose Fast Flow gel column migrated as a single band on SDS–PAGE (Fig. 2).

Ribonuclease activity assay

According to the three-dimensional structures known to date [22,23], the E147 and Y149 residues are located at two opposing sides of the long C-terminal helix of the molecule, while the E96 is located with a rather long distance at the N-terminus of β-strand IV. These three amino acids were predicted as the activity site because

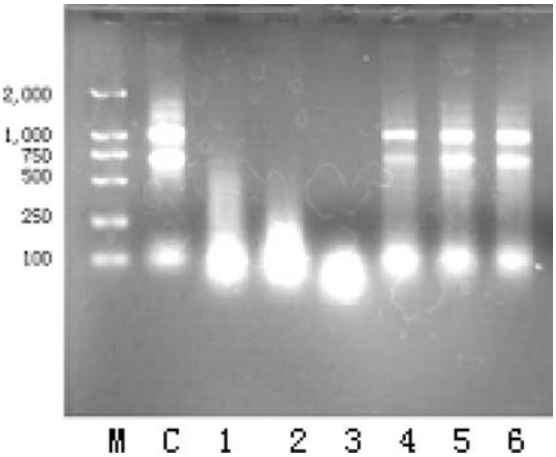

Fig. 3. Ribonuclease activity determination of SPE-16. Lane M: molecular weight marker (bp). Lanes C: RNA after 1 h incubation as the control, 1–6: RNA (6 μg) + native SPE-16 (4 μg), recombinant fusion SPE-16, E95A, E147A, Y149A, and P-loop deletion with 1 h incubation, respectively.

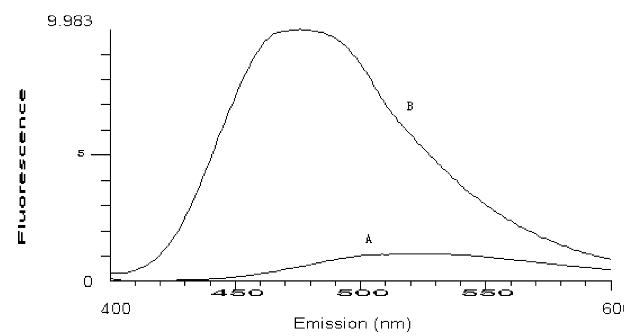

Fig. 4. ANS-binding activity of SPE-16. Fluorescence of 6 μM ANS in the absence (A) and presence (B) of 1 μM SPE-16 in 50 mM Tris–HCl, pH 7.5.

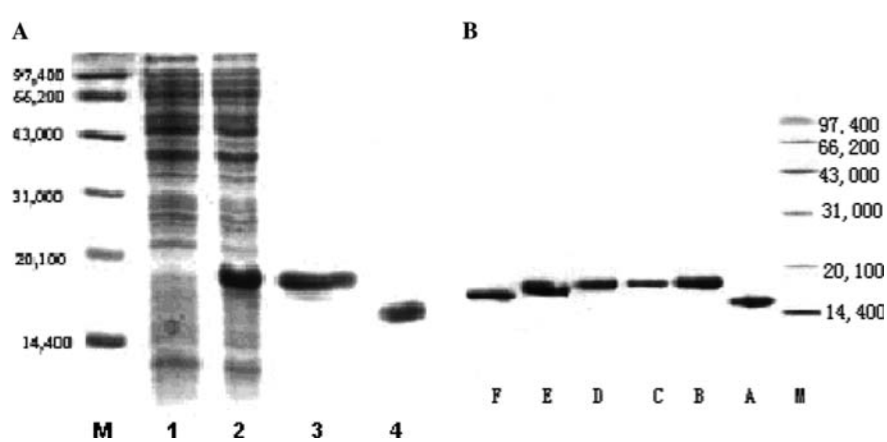

Fig. 2. (A) Purification of SPE-16 from seeds of *P. erosus* and the recombinant expression and purification. Lanes: M, protein marker; 1, nun-induced *E. coli* BL21 (DE3) carrying the SPE-16-insert in pET15b; 2, cells after induction with 1 mM IPTG (3 h, 37 °C); 3, SPE-16 purified fusion protein; and 4, purified native SPE-16 protein. (B) Purified SPE-16 and its mutants. Lanes: M, protein marker; A, native SPE-16; B, recombinant SPE-16; C–F, E95A, E147A, Y149A, and P-loop deletion, respectively.

their side chains have functional groups presumably involved in the catalytic reaction. The in-gel activity assay of native, recombinant, mutant, and truncated SPE-16 proteins showed different effects on ribonuclease activities. The recombinant SPE-16 and mutant E95A kept ribonuclease activity. Mutants E147A, Y149A, and P-loop deleted SPE16 obviously decreased the ribonuclease activity (Fig. 3). These results were similar to those of studies on GaPR10. The replacement of E96 to K96 in GaPR10 showed a 55% decrease of this protein's RNase activity while the E148K and Y150F mutants lost most of their activity [20]. In all of ribonuclease-like PR10 proteins E147 and Y149 are strictly conserved. It has been revealed that the C-terminal helix could be involved in ribonuclease activity of PR10 proteins [22,24]. Another sequence element predicted to be associated with ribonuclease-like activity is the P-loop (GXGGXGXXK), which is involved in ATP or GTP

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PR10.1C         MGVFSFEEETISIVAPSKLFKALTKDSDEIIPKVI-EPIQSVEIVEGNGGPGTIKKIT-A 58
LIPR10.1B       MGVFAFEDEHPSAVAQAKLFKALTKDSDDIIPKVI-EQIQSVEIVEGNGGPGTVKKIT-A 58
LIPR10.1A       MGIFAFENEQSSTVAPAKLYKALTKDSDEIVPKVI-EPIQSVEIVEGNGGPGTIKKII-A 58
ABR17           MGVFVFDDEYVSTVAPPKLYKALAKDADEIVPKVI-KEAQGVEIIEGNGGPGTIKKLS-I 58
SPE-16          -GVFVFRDETSSSVAPAKLYKALTKDSDTIAQKID-GPIQSIELVEGNGGVGTIKKIT-A 57
LaPR10          -GIFTFEDESTSTVAPAKLYKALVADANIIIPKAV-EAIQSVENVEGNGGPGTIKKLT-F 57
PR10.2A         MGVFTFEDESTSTIAPARLYKALVKDADAIIPKAV-EAIQSIETVEGNGGPGTIKKLT-L 58
YPR10           MAVFTFEDQTTSPVAPATLYKALVKDADNIVPKAV-DSFKSVEIVEGNGGPGTIKKIS-F 58
MSPR10-1        MGVFNFEDETTSIVAPARLYKALVTDSDNLIPKVI-DAIQSIEIVEGNGGAGTIKKLT-F 58
GinsengRNase    -GVQKTEVEATSTVPAQKLYAGLLLDIDDILPKAFPQAIKSSEIIEGDGGVGTVKLVT-L 58
Betv1           -GVFNYETETTSVIPAARLFKAFILDGDNLFPKVAPQAISSVENIEGNGGPGTIKKIS-F 58
GaPR10          -GVVSYEFEVTSPIAPARLGKAFVLEAAKIWPTAAPHAVKSVE-LEGDASPGSIVKITTF 58
                 .:     :  * :.   *  .:  :   :  .      .. * :**:.. *::  :

PR10.1C         VHGGHTSYVLHKIDAIDEASLTYDYSIVGGTGLDESLEKITFESKIFSGPDGGSIGKINV 118
LIPR10.1B       SHGGHTSYVLHKIDAIDEASFEYNYSIVGGTGLDESLEKITFESKLLSGPDGGSIGKIKV 118
LIPR10.1A       IHDGHTSFVLHKLDAIDEANLTYNYSIIGGEGLDESLEKISYESKILPGPDGGSIGKINV 118
ABR17           LEDGKTNYVLHKLDAVDEANFGYNYSLVGGPGLHESLEKVAFETIILAGSDGGSIVKISV 118
SPE-16          NEGDKTSFVLQKVDAIDEANLGYDYSIVGGTGLPESLEKLSFETKVVAGSGGGSISKVTL 117
LaPR10          IEDGETKYVLHKIEEIDEANLGYNYSIVGGVGLPDTVEKITFETKLVEGVNGGSIGKVTI 117
PR10.2A         IEGGETKYVLHKIEAVDEANLRYNYSIVGGVGLPDTIEKISFETKLVEGANGGSIGKVTI 118
YPR10           LEDGETKFVLHKIEGIDEANLGYSYSIVGGAALPETAEKITIDSKLSDGPNGGSVVKLSI 118
MSPR10-1        VEGGETKYDLHKVDLVDDVNFAYNYSIVGGGGLPDTVEKISFESKLSAGPDGGSTAKLTV 118
GinsengRNase    GEASQFNTMKQRIDAIDKDALTYTYSIIGGDILLDIIESIVNHFTIVPTPDGGSIVKNTT 118
Betv1           PEGFPFKYVKDRVDEVDHTNFKYNYSVIEGGPLGDTLEKISNEIKIVATPDGGSILKISN 118
GaPR10          VEGLPYQYMKHQIGGHDENNFSYSYSMIEGGPLGDKLEKISYENQFVAAADGGSICKSSI 118
                 .    .   .::   *.  : * **:: *  * :  *.:  .  .    .***  * .

PR10.1C         KFHTKGDV-LSDTVREEAKFKGIGLFKAVEGYVLANPNY-- 156
LIPR10.1B       KFHTKGDV-LSDAVREEAKARGTGLFKAVEGYVLANPNY-- 156
LIPR10.1A       KFHTKGDV-LSETVRDQAKFKGLGLFKAIEGYVLAHPDY-- 156
ABR17           KYHTKGDAALSDAVRDETKAKGTGLIKAIEGYVLANPGY-- 157
SPE-16          KFHTKGDAPLSDAVRDDALAKGAGFFKAIETYL-------- 150
LaPR10          KIETKGDAKPNEQEGKAAKARGDAFFKAIETY--------- 149
PR10.2A         KIETKGDAQPNEEEGKAAKARGDAFFKAIENYLSAHPEYN- 158
YPR10           KYHSKGDAPPNEDELKTGKAKSDALFKVIEAYLLANA---- 155
MSPR10-1        KYFTKGDAAPSEEEIKGGKARGDGLFKALEGYVLANPDY-- 157
GinsengRNase    IYNTIGDAVIPEENIKDATEKAGLIFKAVEAYLLAN----- 154
Betv1           KYHTKGDHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN 159
GaPR10          KYYTVGDYVITEDEIKTLIKGSEVVYKAIEAYLLANPDACN 159
                   : **        .     .  . :.:* *
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Fig. 5. Amino acid sequence alignment of SPE-16 with the other members of the PR-10 family. The identical, highly conserved, and conserved amino acids among the sequences are denoted with (*), (:), and (.), respectively. Predicted activity sites E95, E147, Y149, and P-loop were bolded and underlined.

binding. Our experiments are the first to report the affinity of P-loop deletion to its activity. The P-loop motif is conserved in reported ribonuclease-like PR10 proteins but it varies in GaPR-10 (GDASPGSIVK). Mutations of G51A and K55N in GaPR-10 P-loop also lowered the RNase activity of this protein [20]. These suggested the critical role of P-loop in ribonuclease-like activity of PR10 proteins as predicted.

ANS-binding activity

ANS binding to native SPE-16 can be detected by the appearance of a characteristic spectrum with the fluorescence intensity increased at 477 nm and λ_{max} of ANS was blue-shifted from 530 to 477 nm in less than 10 s (Fig. 4). The fluorescence at different pH showed the strongest binding occurred at pH 4.0.

Discussion

In China, farmers use the powder of *P. erosus* seeds as a pesticide against pest in farmland. And interestingly, vermin were never found in *P. erosus* seeds, suggesting that certain special substances in the seeds are involved in plant defense machinery. The cDNA sequence and the activity assays have further proved that SPE-16 is a PR10 protein with ribonuclease-like activity and ANS-binding activity. Although many PR10 proteins are inducible by pathogen invasion or other elicitors, SPE-16 seems to exist as a constitutive protein and be accommodated with plant developmental regulation.

Fig. 5 shows the sequence alignment of SPE-16 and other PR10 class proteins. Comparison of the amino acid sequence reveals that SPE-16 has sequence identity from 33% to 66% with other reported PRs. The definitive biological function of PR10 family has not been fully determined. But sequence similarity between ginseng RNase 1 with PR10 proteins suggests they have ribonuclease activity. Up to now only Bet v1, Ginseng RNase 1, GaPR-10, LlPR10.1B and LaPR-10, and SPE-16 have been reported to show ribonuclease-like activity in vitro. YPR10 suspected as cytosolic ribonuclease could degrade mRNA induced during stress or pathogen attack in vivo [8], while LlPR10.1A did not demonstrate this activity in vitro [23]. PR10.1C, ABR17, PR10.2A, and MSPR10-1 did not have the correspondent activity reports.

The fluorescence dye 8-anilino-1-naphthalenesulfonate (ANS) is widely used as a hydrophobic probe for proteins [27]. ANS is nonfluorescent in polar solutions while it is brilliantly fluorescent in nonpolar environments. Binding of ANS to proteins ascribes to both its apolar anilinonaphthalene and the negatively charged sulfonate groups. But the fluorescent production due to ANS's anilinonaphthalene group located in a hydrophobic pocket exists in the protein so that it is completely prevented from water quenching and therefore fluorescent [28]. The fluorescent experiment demonstrated SPE-16 could bind ANS in the native state. This result and its homology with both Bet v1 and Lupin extrapolated to mean that SPE-16 also had this hydrophobic cavity. Recent work did by Markovic-Housley et al. [29] also showed that a 30 Å deep Y-shaped hydrophobic pocket spans Bet v1l binded apolar ligands deoxycholate whose structure is similar to those of plant steroid hormone brassinosteroids. This finding indicated that the hydrophobic pocket can bind and transport plant hormones, which are involved in plant defense as well as its growth and development. In vitro activity assay showed SPE16 had both ribonuclease-like activity and hydrophobic molecular-binding function. Which one is more critical for its biological function is still unknown. Current evidence could not rule out the possibility that SPE-16 and Bet v1 [19,26] are bifunctional proteins, whose ribonuclease-like activity is responsible for self-defense mechanisms whereas its ANS-like molecule-binding activity corresponds to phytohormone binding and transport function required by plant growth and development.

Acknowledgments

This work is supported by the Foundation for Author of National Excellent Doctoral Dissertation of P.R. China (Project No. 07101001), the National High Technology Research and Development Program of China (Grant No. 2001AA233021), the 863 Special Program of China (Grant No. 2002BA711A13), the National Natural Science Foundation of China (Grant No. 30121001), and National Foundation of Talent Youth (Grant No. 30225015).

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