

การตรวจหาโปรตีนที่มีฤทธิ์ยับยั้งไรโบโซมในพืชวงศ์ข้าว

Identification of Ribosome-Inactivating Protein-Encoding Genes in the Poaceae Family

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Abstract

Ribosome-inactivating proteins (RIPs), which have been reported from many plants, are an RNA N-glycosidase that depurinates the glycosidic bond of 28S rRNA, thus damaging ribosomes and arresting protein synthesis. The plants produce these proteins to protect themselves from bacterial, viral and fungal infection. Recently, many studies aim to elucidate the three dimensional structures of RIPs and to study their biological activities including antiviral and anticancer properties. The study in relationship and distribution of RIPs in many plants implies that there is abundant distribution of the proteins in nature. Therefore, we can use the information to assess novel proteins. In this study, we have determined RIPs-encoding genes in sugar cane (*Saccharum officinarum*) and cogon grass (*Imperata cylindrica*), which belong to the Poaceae family. We found that using Polymerase Chain Reaction technique and DNA sequencing analysis, the RIPs-coding genes were identified in sugar cane and cogon grass and their sequences were homologous to those of rice (*Oryza sativa*), barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*). Our results suggested that the plants possibly consist of several novel proteins that can be potentially developed as therapeutic agents.

Keywords: Ribosome-inactivating protein, *Saccharum officinarum*, *Imperata cylindrica*, Poaceae family

บทคัดย่อ

โปรตีนที่มีฤทธิ์ยับยั้งไรโบโซม (RIPs) มีฤทธิ์เป็นเอนไซม์ RNA N-glycosidase ซึ่งจะตัดหมู่ฟิวรีนออกจากพันธะไกลโคซิดิกของ 28S rRNA ซึ่งทำให้ไรโบโซมเสียหายและการสังเคราะห์โปรตีนถูกยับยั้ง RIPs สามารถพบได้ในพืชหลายชนิด เช่น ricin จากกะหล่ำหูก MAP30 จากมะระ และ abrin จากมะกล่ำตาแดง ในปัจจุบันนี้มีการศึกษาเกี่ยวกับ RIPs มากมายทั้งเพื่อค้นหาโปรตีนใหม่ ศึกษาโครงสร้างสามมิติและศึกษาฤทธิ์ทางชีววิทยา เช่น ฤทธิ์ต้านเชื้อไวรัสและฤทธิ์ต้านมะเร็ง จุดประสงค์ของการศึกษาค้นคว้าครั้งนี้เพื่อตรวจหายีนที่ทำหน้าที่สร้าง RIP จากอ้อยแดง (*Saccharum officinarum*) และหนุ่ (*Imperata cylindrica*) ซึ่งเป็นพืชในวงศ์ข้าว (Poaceae family) โดยใช้เทคนิคพีซีอาร์และการวิเคราะห์ลำดับดีเอ็นเอ พบว่ายีน RIP ที่พบในอ้อยแดงและหนุ่มีความคล้ายคลึงกับยีน RIPs ที่พบในพืชวงศ์ข้าวได้แก่ ข้าวเจ้า (*Oryza sativa*) ข้าวบาเลย์ (*Hordeum vulgare*) และข้าวสาลี (*Triticum aestivum*) จึงสามารถสรุปได้ว่าอ้อยแดงและหนุ่ค่านั้นน่าจะมียีนที่ทำหน้าที่สร้างโปรตีนที่มีฤทธิ์ยับยั้งไรโบโซมและน่าจะมียีนภาพในการพัฒนาเป็นยารักษาโรคต่อไป

คำสำคัญ: โปรตีนที่มีฤทธิ์ยับยั้งไรโบโซม อ้อยแดง หนุ่ พืชวงศ์ข้าว

Introduction

Many plants contain proteins that are capable of inactivating ribosomes. Accordingly the proteins are called ribosome-inactivating proteins or RIPs. RIPs are RNA N-glycosidase enzymes which damage ribosomes in an irreversible manner. RIPs remove a site-specifically single adenine residue (A4324 in the case of rat liver ribosome) from the highly conserved sarcin/ricin loop of the 28S rRNA, thus arresting protein synthesis (Barbieri et al, 1993; Endo et al, 1989). RIPs are conventionally classified into three types: type 1, 2 and 3 based on the conformation of their subunits (Nielsen and Boston, 2001). While, type 1 RIPs are single-chained proteins with enzymatic, type 2 RIPs are composed of two chains (chain A and

B). Chain A possesses the RIP activities similar to type 1 RIPs and chain B is basically a galactose-binding lectin, which facilitates cell surface binding and internalization of the two-chain molecule (Sandvig and van Deurs, 2002). While, type 3 RIPs, which are identified mostly in maize and barely, consist of an N-terminal active chain linked to an unrelated C-terminal domain with unknown function. RIPs are considered to be plant defense-related proteins as they are able to inhibit the multiplication and growth of several pathogenic virus, fungi, and bacteria either alone or in combination with other defense-related proteins. Furthermore, they have also been used to develop as therapeutic agents with antiviral, anticancer or immuno-modulating properties.

RIPs were initially isolated in plants, mostly in Angiospermae, both monocotyledons and dicotyledons, and also in mushroom, alga, bacteria and fungi (Barbieri et al, 1993). Type 1 RIPs are widely discovered more frequent than type 2 and type 3 RIPs, and seem to be preferentially widespread among plants belonging to some families such as Cucurbitaceae, Caryophyllaceae, Euphorbiaceae, Amaranthaceae, Poaceae and others. From plants of the Poaceae family, both the type 1 and type 3 have been identified: such as rice (*Oryza sativa*), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*) and corn (*Zea mays*). For example of type 1 RIP, Tritin and Barley RIP have been identified in *T. aestivum* and *H. vulgare*, respectively (Hartley, Chaddock and Bonness, 1996). Type 3 RIPs found in the Poaceae family were such as maize protein b-32 and barley RIP, JIP 60 (Stirpe and Battelli, 2006). In our present study, we attempted to identify RIPs-encoding gene in sugar cane (*Saccharum officinarum*) and cogon grass (*Imperata cylindrica*) by using polymerase chain reaction (PCR) technique and DNA-sequencing analysis.

Materials and Methods

1. Materials

Plant materials: Fresh plant leaves of sugar cane and cogon grass were collected from Suphanburee province, Thailand during July, 2004. The young leaves were used as the source of plant genomic DNA.

Synthetic oligonucleotide: Synthetic oligonucleotides were purchased from the Bio-service Unit, the National Center for Genetic Engineering and Biotechnology (BIOTECH), The National Science and Technology Development

Agency (NSTDA), Bangkok, Thailand. The primers were synthesized based on the nucleotide sequence of RIPs gene from the Poaceae family.

Enzyme: *Taq* DNA polymerase (SibEnzyme, USA) is capable of adding non-templated adenine residue at 3' end of a DNA fragment. For this reason PCR products produced with this enzyme can be use in UA cloning.

Cloning vector: pDriveTM (QIAGEN, USA) used as a cloning vector is the linear from with a U overhang at each end. This vector contains both ampicillin and kanamycin resistance genes as well as a blue-white selection site. Additionally, the vector contains a T7 promoter and SP6 promoter on either side of the cloning site, allowing sequence analysis using standard sequencing primers.

Bacterial strain: *Escherichia coli* strain DH5 α .

Culture medium: LB broth medium (Luria-Bertani broth) contained 1% w/v bacto tryptone, 0.5% w/v yeast extract and 1% w/v NaCl adjusting pH to 7.0 with 1 N NaOH. For LB agar medium, the ingredient of the media was exactly the same as LB broth but the bacto agar is added to 1.5% w/v.

Chemical reagents: All the chemical reagents used in this study were the analytical grade or molecular biological grade from various suppliers.

2. Methods

Isolation of plant genomic DNA, The young fresh leaves of rice, cogon grass and sugar cane were used for isolation of genomic DNA using the procedure described by Rogers

and Bendich (Rogers & Bendich, 1994). The plant leaves were collected and washed with distilled water, and aliquots of 200 mg of plant tissue were stored at -70°C freezer for further use. For the genomic isolation step, the plant tissue was grinded on dry-ice using -20°C pre-cooled mortar and pestle. The powder was transferred to 1.5 mL microtube and allowed the dry-ice to evaporate on an ice box. Next, 0.6 μL 2x CTAB buffer (2% CTAB, 1% PVP, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl pH 8.0, 0.2% 2-mercaptoethanol) was pre-heated at 60°C and then added to the tissue powder and further incubated at 60°C for 30 minutes with gentle shaking every 15 minutes. The cell debris was removed by centrifugation at 14,000 rpm for 10 minutes. The supernatant was treated with an equal volume of chloroform:isoamyl alcohol solution (24:1) for 10 minutes and mixed several times by inversion. The mixture was centrifuged at 14,000 rpm for 10 minutes. The aqueous phase was transferred to a new 1.5 mL microtube, added with 5 μL RNase A solution (10 mg/mL), incubated at 37°C for 30 minutes and then treated with an equal volume of chloroform:isoamyl alcohol solution (24:1) for 10 minutes again. Then, the aqueous phase was transferred to a new 1.5 mL microtube, added with equal volume of pre-cooled isopropanol, and incubated at -20°C overnight. The DNA pellet was collected by centrifugation for 10 minutes at 14,000 rpm and washed with 70% ethanol. The DNA pellet was allowed to dry at room temperature, re-suspended in TE buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA) and analyzed by agarose gel electrophoresis. The DNA solution was stored at -20°C for further use.

Agarose gel electrophoresis: Genomic DNA and plasmid DNA was analyzed on 0.8% agarose gel (0.8% SeaKemTm LE agarose in 0.5X TBE buffer: 44.5 mM Tris-HCl pH 7.4, 44.5 mM boric acid and 20 mM EDTA) to determine their size and concentration. In case of PCR fragment analysis, 1.2% agarose gel was used. DNA samples were mixed with DNA loading dye (25% glycerol, 60 mM EDTA, 0.25% bromophenol blue) in ratio of 3:1 and loaded into well. Electrophoresis was performed at 100 volts for 45 minutes. DNA bands were analyzed by 25 $\mu\text{g/mL}$ ethidium bromide staining and visualized under a UV lamp.

PCR amplification from genomic DNA: Polymerase chain reactions (PCR) were performed using a pair of primers. The primers were designed according to *RIPs*-encoding genes from the Poaceae family. The PCR was amplified by using genomic DNA as template and POAF (ACA ACC TCT ACC TGG AGG GAT TC) and POAR (ACG AAC AGC AGG ATC CCC A), as forward and reward primers, respectively. Twenty five μL PCR reaction consists of 100-200 ng of DNA template, 1 unit of DNA polymerase, 0.8 pM primers, 1X buffer supplied with enzyme, 2.5 mM MgCl_2 , 0.4 mM dNTPs and sterilized distilled water for adjust volume. The PCR was performed in a thermocycler (Tpersonal, Biometra®) pre-heated at 96°C done for 5 minutes before starting with 35 cycles of denature temperature of 96°C for 1 minute, annealing temperature of 55°C for 1 minute 15 seconds, and extension temperature of 72°C for 1 minute. After 35 cycle amplifications, the final extension at 72°C was performed for 7 minutes and PCR products were analyzed on 1.2% agarose gel.

DNA purification: After the amplification, the PCR products were resolved by 0.8% agarose gel electrophoresis. The PCR products were purified from gel by using QIAquick™ Gel Extraction kit (QIAGEN®) and the purified products were used at the ligation reaction step.

Cloning of PCR fragment into cloning vector: The purified band of interest was ligated to the linear cloning vector, pDrive™. Ligation of fragment with pDrive™ was performed on ice box for 3 hours. Five µL of reaction mixture contained 25 ng of pDrive™ cloning vector, 2 µL of purified PCR product and 1X ligation buffer. The ligation product was transformed to the *E. coli* DH5α competent cells and the positive clones were selected by white colony selection with the ampicillin resistance phenotype. In addition, the positive clones were confirmed by PCR amplification.

Screening of the positive clones by PCR amplification from bacterial culture using the colony PCR method: Fresh single colonies from the culture plate was individually scraped with a toothpick and resuspended into 10 µL of TTE buffer (1% tritonX-100, 1 M Tris-HCl pH 8.0 and 0.5 M EDTA). The suspension was boiled for 10 minutes to obtain cell lysate. The cell debris was removed by centrifugation at 12,000 rpm for 5 minutes. Finally, 2.5 µL of supernatant was used as PCR template and PCR amplification were carried out as previously described.

Miniprep plasmid DNA isolation by CTAB method: One single colony of bacterial was grown in 3 mL of LB broth with ampicillin supplement to final concentration of 100 µg/mL. Cells were harvested by centrifugation for 3

minutes at 14,000 rpm for 1 minute. Cell pellet was resuspended in 200 µL STET buffer (8% w/v sucrose, 0.1% v/v tritonX-100, 50 mM EDTA and 50 mM Tris-HCl pH 8.0) and incubated with 5 µL lysozyme solution (50 mg/mL) for 10 minutes at room temperature. The reaction was stopped by boiling for 45 seconds and cell debris was removed by centrifugation at 14,000 rpm for 15 minutes. The 1/10 volume of 5% CTAB was added to the supernatant and the mixture was mixed by gentle rocking to allow precipitation of plasmid DNA. The DNA pellet was resuspended with 300 µL of 1.2 M NaCl and treated with 5 µL of 10 mg/mL RNase A at 37 °C for 15 minutes. Next the chloroform extraction was performed and the plasmid DNA was precipitated by adding an equal volume of pre-cooled absolute ethanol. The plasmid DNA was allowed to precipitate overnight at -20 °C. Finally, the DNA pellet was washed with 70% ethanol and dissolved in TE buffer.

DNA sequencing: The PCR fragment in pDrive™ was sequenced on both strands using the T7 promoter primer and SP6 promoter primers. The sequencing process was performed by Bio-service Unit, the National Center for Genetic Engineering and Biotechnology (BIOTECH), the National Science and Technology Development Agency (NSTDA), Bangkok, Thailand.

Computational analyses: The primers, POAF and POAR were designed based on the RIPs-encoding gene from *O. sativa*, *H. vulgare* and *T. aestivum* using Primer 3 program. DNA sequence data were analyzed with Clustal X. Multiple sequence alignment was performed with Clustal X. Identity of nucleotide sequences were determined using Bio Edit software version 5.0.9.

Pair wise matrix comparing amino acids were analyzed using Clustal W. Alignment of deduced amino acid for several cloned RIPs were performed with Clustal W.

Results

The young leaves of rice (*O. sativa*), cogon grass (*I. cylindrical*) and sugar cane (*S. officinarum*) were used as the source of genomic DNA. The fragments of *RIPs*-encoding genes were successfully amplified by using genomic DNAs with primers POAF and POAR. The approximately 500-bp fragments were visualized on agarose gel (Figure 1). Using Clustal X, the nucleotide sequences of *I. cylindrical* and *S. officinarum* were aligned and compared with the *RIP*-encoding sequences reported in the GenBank database as shown in Figure 2. The identities of nucleotide sequences of the Poaceae family were shown in Table 1. The result from the nucleotide sequence alignments of the putative *RIP* genes from *I. cylindrical* and *S. officinarum* with other type 1 *RIPs* from Poaceae family indicated that these

two *RIP*-like genes had nearly 66-78% identity to other type 1 *RIPs* from the Poaceae family. However, the comparison of the two nucleotide sequences of *S. officinarum* and *I. cylindrical* displayed 98% identity.

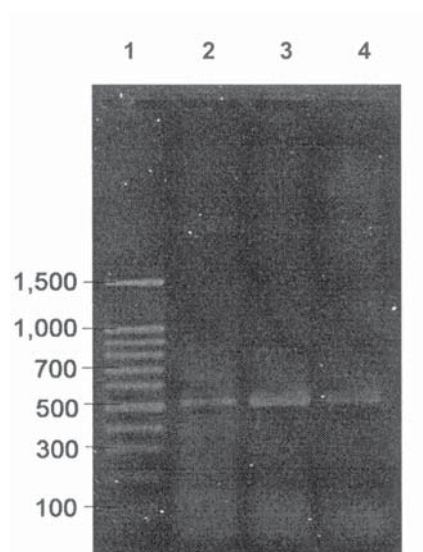


Figure 1 PCR products of the amplified fragment of *RIPs*-encoding genes. Lane 1; 100 ng of DNA 100 bp ladder marker. Lane 2-4; 4 μ L PCR products of *O. sativa*, *I. cylindrical* and *S. officinarum*, respectively.

Table1 Identities of the *RIP*-encoding genes in the Poaceae family.

	<i>S. officinarum</i>	<i>I. cylindrical</i>
<i>O. sativa</i> RIP1 (AB051107)	0.78	0.77
<i>O. sativa</i> RIP2 (AB051108)	0.67	0.66
<i>H. vulgare</i> (M62905)	0.77	0.76
<i>T. aestivum</i> (D13795)	0.77	0.77

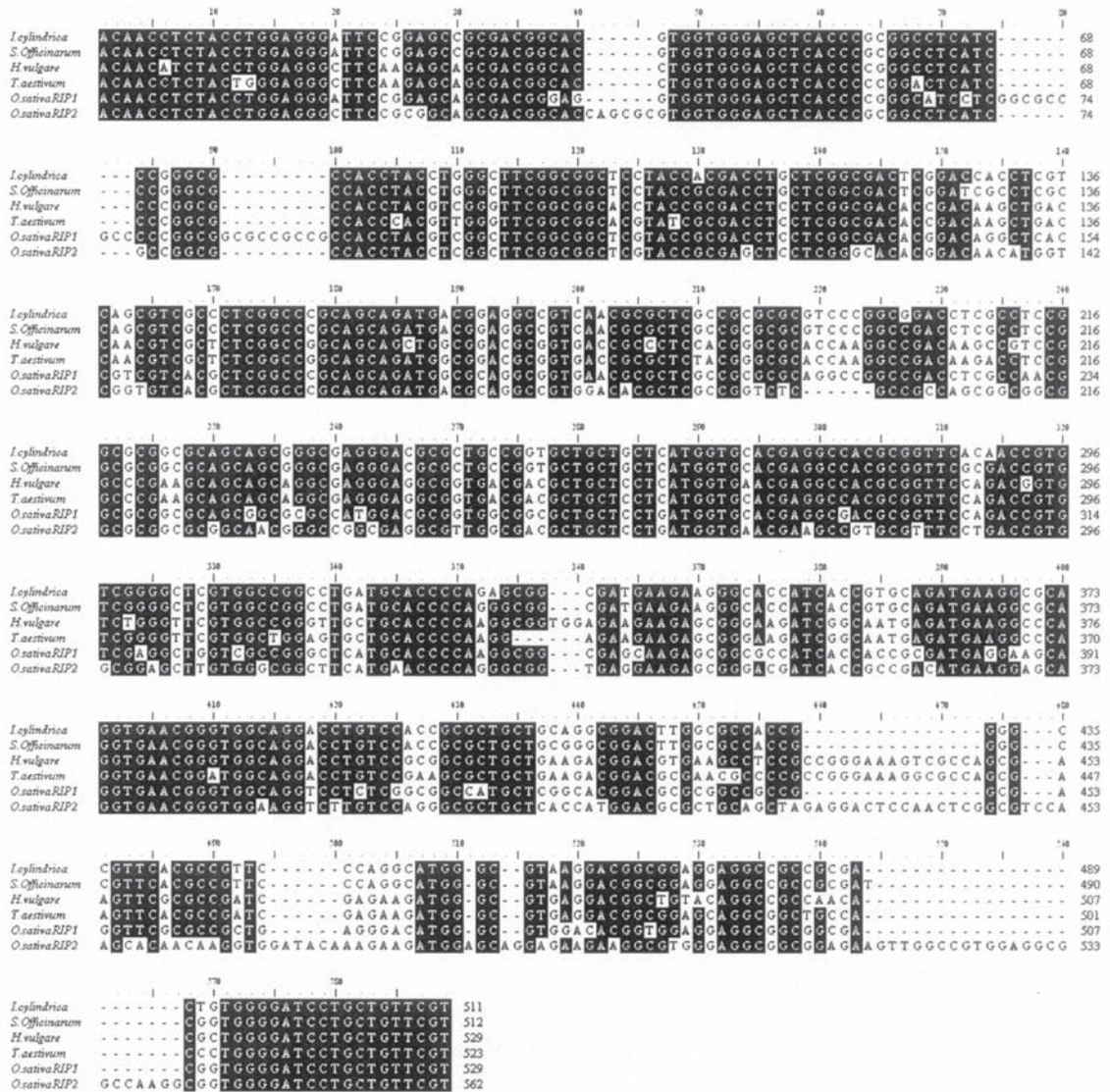


Figure 2 The nucleotide sequence alignments *RIPs*-encoding genes in the Poaceae Family. *RIPs* from *H. vulgare* (M62905), *T. aestivum* (D13795), *O. sativa* RIP1 (AB051107), *O. sativa* RIP2 (AB051108), *S. officinarum* and *I. cylindrica* were used for the sequence comparison.

Pair wise matrices comparing the amino acid sequences of our putative *RIPs* genes with other type 1 *RIPs* from Poaceae family is shown in Figure 3. The upper matrix specifies identity values between the sequences, while the lower matrix shows a comparison of the similarity values between those sequences. The gene from *I. cylindrica* showed maximum similarity of 94%

and maximum identity of 93% with the one from *S. officinarum*.

Alignment of the deduced amino acid sequences of the putative *RIPs* with the related type 1 *RIPs* from the Poaceae family and the A chain of ricin revealed the presence of 25 conserved residues (Figure 4). The underline region indicated the active site of *RIPs*.

Identity							
	<i>I. cylindrica</i>	<i>S. officinarum</i>	<i>T. aestivum</i>	<i>H. vulgare</i>	<i>O.sativa</i> RIP1	<i>O.sativa</i> RIP2	Ricin
<i>I.cylindrica</i>		0.93	0.67	0.65	0.70	0.57	0.19
<i>S. officinarum</i>	0.94		0.65	0.63	0.68	0.51	0.18
<i>T.aestivum</i>	0.77	0.74		0.90	0.63	0.51	0.17
<i>H.vulgare</i>	0.76	0.73	0.94		0.63	0.51	0.17
<i>O.sativa</i> RIP1	0.77	0.74	0.73	0.74		0.51	0.17
<i>O.sativa</i> RIP2	0.64	0.60	0.61	0.63	0.62		0.15
Ricin	0.37	0.34	0.35	0.36	0.35	0.33	
Similarity							

Figure 3 Pair wise matrices comparing the amino acid sequences of RIPs from a series of plant species. The upper matrix specifies identity the values between the sequences, while the lower matrix is a comparison of the similarity values between those sequences. Clustal W program was used to generate the matrix. The RIPs from *H. vulgare* (M62905), *T. aestivum* (D13795), *O. sativa* RIP1 (AB051107), *O. sativa* RIP2 (AB051108), *S. officinarum* and *I. cylindrica* were compared.

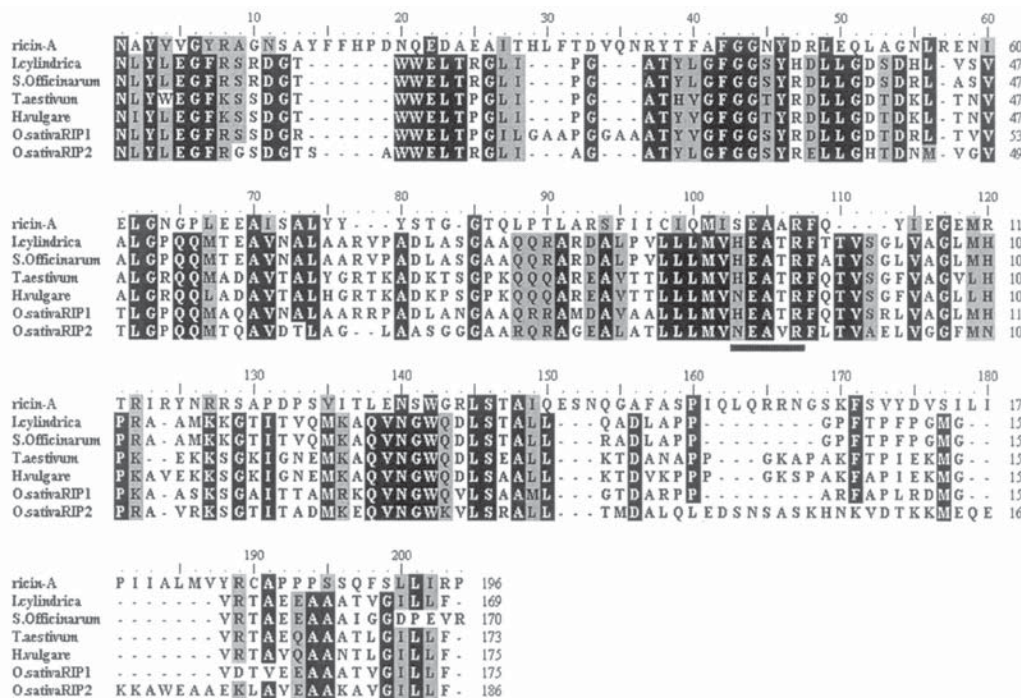


Figure 4 Comparison of the deduced amino acid sequence of RIPs gene in the Poaceae family. RIPs from *H. vulgare* (M62905), *T. aestivum* (D13795), *O. sativa* RIP1 (AB051107), *O. sativa* RIP2 (AB051108), *S. officinarum* and *I. cylindrica* were compared. Identical residues are highlight with black box. The underline region indicated the active site identified in RIPs.

Discussion

As expected, the size of the PCR product containing the partial fragment of RIPs gene is approximately 500 bp long. Therefore, the pair of primers (POAF and POAR) can be used for the identification of the RIPs gene in the Poaceae family.

The genomic DNAs extracted from young leaves of *O. sativa*, *I. cylindrical* and *S. officinarum* were used for the template of the PCR amplification with the same pair of primers. Lane 2-4 (Figure 1) on the gel electrophoresis show the strong approximately 510 bp bands, which have the same size as previously reported by PCR amplification of the genomic DNA of *O. sativa*. Finally, the data from nucleotide sequence alignment of two fragments with the others Poaceae family clearly indicated that the sequences are high homologous. As show in type 1 of Cucurbitaceae family, the nucleotide sequences alignment of type 1 RIPs had nearly 30-80% identity with other (Chuethong, 2007; Sornlek 2005; Watthanachaiyingcharoen, 2002).

Conclusion

At the present study, we are able to amplify a gene fragment possibly coding for the novel Ribosome-inactivating proteins from sugar cane (*S. officinarum*) and cogon grass (*I. cylindrical*). The nucleotide sequences of these fragments are high homologous to RIPs-genes of rice (*O. sativa*), wheat (*T. aestivum*) and barley (*H. vulgare*). Comparison of the two new nucleotide sequences show about 66 - 78% of identity with ones from other Poaceae family recorded in GenBank.

Our finding from this study suggests that both cogon glass and sugar cane contain novel RIPs-like encoding genes. We are in the process of the isolation of the complete genes and the further study are to asses the biological activities of the new RIPs before being developed as therapeutic agents.

References

- Barbieri L, Battelli MG, Stirpe F. 1993. Ribosome-inactivating proteins from plants. *Biochem Biophys Acta* 1154: 237-82.
- Chuethong J. 2007. Cloning, expression and biological properties of type 1 ribosome-inactivating protein from the seeds of spiny bitter cucumber (*Momordica cochinchinensis*) (Dissertations). Faculty of Graduate Studies, Mahidol University.
- Hartley MR, Chaddock JA, Bonness MS. 1996. The structure and function of ribosome-inactivating proteins. *Trends in Plant Sci.* 1: 254-260.
- Nielsen K, Boston RS. 2001. Ribosome-inactivating proteins: A plant Perspective. *Annu Rev Plant Physiol Plant Mol Biol* 52: 785-816.
- Rogers SO, Bendich AJ.1994. Extraction of total cellular DNA from plants, algae and fungi In: Plant Molecular Biology Manual. Kluwer Academic Publishers. D1/1-8.
- Sandvig K, van Deurs B. 2002. Transport of protein toxins into cells: pathways used by ricin, cholera toxin and Shiga toxin. *FEBS Lett* 529: 49-53.
- Sornlek P. 2005. Ribosome inactivating protein from plant *Momordica charantia*: cloning, expression and biological assay (Dissertations). Faculty of Graduate Studies, Mahidol University.
- Stirpe F, Battelli MG. 2006. Ribosome-inactivating proteins: progress and problems. *Cell Mol Life Sci*, 63: 1850-1866.
- Watthanachaiyingcharoen R. 2002. Purification and characterization of pharmacologically active proteins from plants in the genus of *Momordica* (Dissertations). Faculty of Graduate Studies, Mahidol University