Article

Gene Isolation Using Degenerate Primers Targeting Protein Motif: A Laboratory Exercise[S]

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Abstract

Structures and functions of protein motifs are widely included in many biology-based course syllabi. However, little emphasis is placed to link this knowledge to applications in biotechnology to enhance the learning experience. Here, the conserved motifs of nucleotide binding site-leucine rich repeats (NBS–LRR) proteins, successfully used for the isolation and characterization of many plant resistance gene analogues (RGAs), is featured in the development of a series of laboratory experiments using important molecular biology techniques. A set of previously isolated RGA sequences is used as the model for performing sequence alignment and visualising 3D protein structure using current bioinformatics programs (Clustal Omega and Argusdock software). A pair of established degenerate primer sequences is provided for the prediction of targeted amino acids sequences in the RGAs. Reverse transcription-polymerase chain reaction (RT-PCR) is used to amplify RGAs from total RNA samples extracted from the tropical wild relative of black pepper, *Piper colubrinum* (Piperaceae). This laboratory exercise enables students to correlate specific DNA sequences with respective amino acid codes and the interaction between conserved motifs of resistance genes with putatively targeted proteins. © 2017 by The International Union of Biochemistry and Molecular Biology, 46(1):47–53, 2018.

Keywords: *Protein motif; degenerate primers; RT-PCR; gel electrophoresis; targeted gene isolation*

Introduction

Proteins are the basic building blocks of life, involved in almost all biochemical reactions in life and are considered as one of the most versatile macromolecules discovered [1]. Proteins can function as a biological catalyst (e.g. amylase), a transportation medium (e.g. haemoglobin), or even as a structural component of mechanical scaffoldings (e.g. skeletal muscle) of an organism. Proteins contain a broad range of functional groups on their amino acids monomers. Thiols, thioethers, carboxamides, alcohol, and carboxylic acids are all commonly present in protein molecules [2]. The arrangement of the functional groups usually determines the final properties of the protein. One interesting group of proteins contains a large number of the hydrophobic functional group that allows them to form a transmembrane protein motif capable of transferring signals from the extracellular matrix into the cell body (Fig. 1) [3]. This is especially important to cells as it facilitates detection of the presence of a pathogen before any infection occur [4].

The structural significance of proteins is included in many science-based educational materials. In the fields of chemistry, biochemistry, and molecular biology, core functions of protein structure are often explained as abstract concepts. Hence, students may often face difficulties in understanding fundamental concepts of protein beyond the commonly used "Locks and Keys" concept [5]. Deeper understanding and a better appreciation of the concepts involved in many protein–protein interactions can be achieved by providing a hands-on experience [6] and relating it to actual research applications.

An important, relevant real-world application involves the detection and identification of conserved protein motifs

Volume 46, Number 1, January/February 2018, Pages 47–53
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[S]Additional Supporting Information may be found in the online version of this article.
Received 4 October 2016; Accepted 2 October 2017
DOI 10.1002/bmb.21089
Published online 13 November 2017 in Wiley Online Library (wileyonlinelibrary.com)

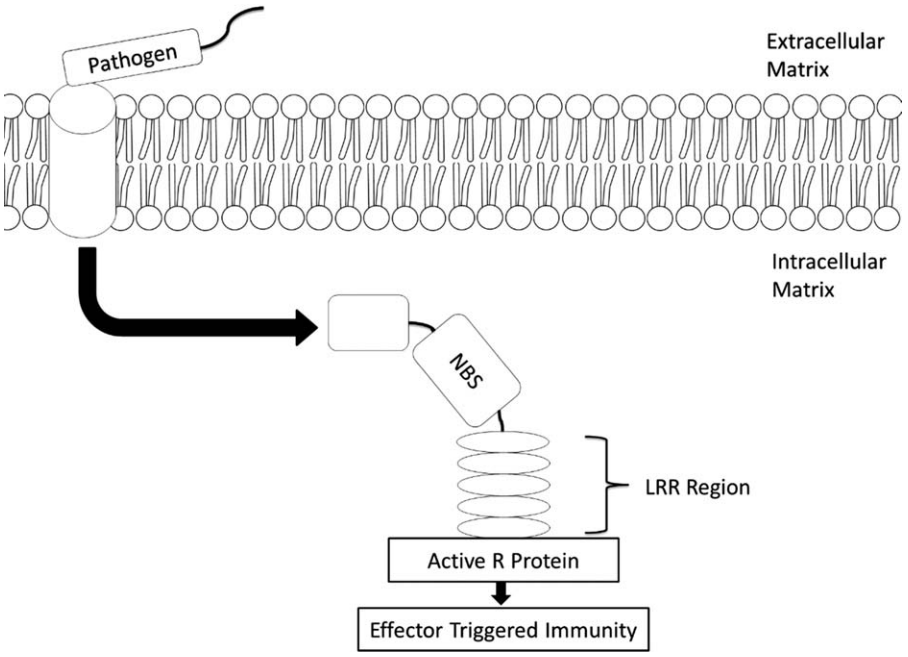

FIG 1

The NBS and LRR detect effector proteins from pathogens. The NBS–LRR protein also often has a TIR or CC domain at its N-terminus important for the activation of the immune response. Infection with pathogens transfers protein-mediated signals into the plant cell which activates the R protein and leads to the activation of effector-triggered immunity. TIR, Toll/interleukin-1 receptor-like domain; CC, coiled-coil domain; NBS, nucleotide binding site; LRR, leucine-rich repeats; R protein, resistance protein. Figure was reconstructed from Spoel and Dong [3].

that correlate with the expression of disease resistance genes in crop plants. There are several motif structures that are responsible for the signal transduction system of plant immune systems [7, 8]. This paper focuses on two conserved protein motifs, the characteristic nucleotide binding site (NBS) motif and the leucine-rich repeats (LRR) motif. The NBS and LRR motifs are connected as a complex that mediates the effector-triggered immunity in plants (Fig. 1). The NBS motif is responsible for the activation of kinases in signal transduction pathways [9] while the LRR domain functions in ligand binding and pathogen recognition of the resistance proteins [10].

Overall sequences among members of the (NBS–LRR) resistance gene family are subjected to many variations and could not be detected directly by cross-hybridization technique. However, short stretches of protein sequences within the gene are well conserved between most resistance genes [8, 9]. These conserved motifs enable the use of polymerase chain reaction (PCR) based technique for the isolation of resistance gene analogues (RGA) from known resistant plants or for use as markers to screen breeding material for potentially resistance/susceptible plants. Utilizing degenerate primers, which are a mixture of primers with different substitutions at selected nucleotides targeted at the NBS domain, is possible to amplify new NBS–LRR genes based on the sequence homology. Here, we report a comprehensive laboratory exercise encompassing both molecular techniques and bioinformatics programs for protein sequence analysis that includes total RNA isolation, PCR amplification of targeted cDNA sequences corresponding to the protein motifs of plant disease resistance genes, and agarose gel visualization of PCR amplicons from the wild relative of black pepper, *Piper colubrinum*.

Background for Experimental Exercise

Piper nigrum, also commonly known as black pepper, is one of the most important spices used in the world. Grown mainly in tropical climates such as in Malaysia, the state of Sarawak accounts for 98% of the total production volume of black pepper in the country. As with many crop plants, plant protection against potential pathogens is an important area of research to minimize infections and ensure stable production. The ability of a plant to recognize pathogen and rapidly initiate proper defence reactions determines the success or failure of a pathogen infection [3]. If the virulence gene on the pathogen is recognized immediately by the corresponding resistance gene in the plant, resistance mechanisms are activated rapidly to protect the plant against infection. However, if the resistance gene is absent in the plant, defence reactions are triggered in a slower manner, thus resulting in pathogenic infections [11].

While *P. nigrum* is more susceptible to infections, *P. colubrinum* (a wild relative of *P. nigrum*) displays a higher degree of resistance towards plant diseases caused by the fungal-like pathogen *Phytophthora capsici* and nematode *Radopholus similis* [12]. *P. colubrinum* was recognized as a high-potential donor plant in breeding programmes for the development of disease-resistance in *P. nigrum* and is part of the strategy to improve both production quantity and quality of *P. nigrum* [13].

Pre-Experimental Exercise 1: Amino Acids Alignment and Protein Modelling

Amino Acids Sequence Alignment via Clustal Omega

Firstly, to illustrate an example of the concept of a protein–protein interaction, a diagram of a plant resistance protein with NBS–LRR domain interacting with a receptor is provided (Supporting Information Material, Exercise A, Q1). Observation of this diagram will facilitate recognition of the NBS domain with the specific structure binding to the receptor.

This is then followed by observations of the NBS–LRR domain from case examples. A multiple sequence alignment exercise using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) [14], a currently available web-based bioinformatics program is performed to analyse five NBS–LRR type resistance genes previously isolated from *P. colubrinum*. These amino acid sequences isolated from *P. colubrinum* are retrieved from the NCBI GenBank database in Fasta format based on the accession numbers given: JN574875.1, JN574876.1, JN574877.1, JN574878.1, and JN574879.1. These selected sequences cover the P-loop [GGVGKTT] motif and the hydrophobic [GLPLAL] motif of the NBS–LRR domain. Results of the multiple sequence alignment demonstrate these key motifs characteristic of resistance proteins. A table summarizing amino acid sequences of the five NBS–LRR type RGA previously isolated from *P. colubrinum* is attached to be used as a reference during sequence retrieval (Supporting Information Material, Appendix, Table 7).

Protein 3D Structure Visualization Using ArgusLab

To further strengthen understanding of protein structures, a molecular modelling software ArgusLab 4.0 (http://www.arguslab.com/arguslab.com/ArgusLab.html) is used to visualize the 3D structural appearance of the NBS and LRR motifs in the plant resistance protein. ArgusLab 4.0 has the advantage of being relatively user-friendly, especially for beginners. It operates on Microsoft Windows platform, which is a common operating system for most institutes' computers.

For this part, students are asked to obtain a specific protein file with the PDB entry name '1Z6T', corresponding to the human apoptosis regulator Apaf-1 from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (PDB) (http://www.rcsb.org/pdb/home/home.do) [15]. This protein file is used for the visualization of the secondary and tertiary structure of an NBS–LRR protein complex in this laboratory exercise. It needs to be explained that at present, no experimentally determined NBS–LRR protein complex of *P. colubrinum* is available in the data bank. However, the conservative nature of the NBS–LRR subdomains shares enough structural homology to fulfil the purpose of 3D visualization of the protein domain. The PDB file '1Z6T', despite its nonplant origin, has been widely used as a template for computational protein structure prediction of the NBS domain system in plants such as *Cocos nucifera* (coconut) [16] and *Curcuma zedoaria* (turmeric) [17].

The NBS motif structure is generally complicated and can be challenging to identify each of the motifs individually on the 3D protein structure. For students introduced to a protein modelling software for the first time, guided learning is implemented in the form of having a list of specific questions to aid and motivate students to navigate through the software and while visualizing the complex protein model (Supporting Information Material, Exercise A, Q1 Fig. 1). Students are requested to compare the structure of this protein (1Z6T) to a typical NBS domain and detect similarities between the two structures. This is done by deleting all the chains using the navigation pane on the software. All the amino acids except those in the "Chain A" folder are selected and removed using the "delete" option in the right-click menu. It is also recommended to display the protein secondary structure in ribbon structure form by selecting View → Hide protein and clicking on the "Render protein as cartoon ribbon" option from the main menu. The secondary structures of the protein are also made clearer (more apparent) by selecting View → "Colour ribbon by secondary structure".

Due to the large diversity of structural sequences within the LRR domain and the ambiguity of the motifs, it is not facile to select an ideal template from the protein databank. For the visualization of LRR motif, the students are instead given several protein templates of LRR containing structures and asked to identify the conserved structure of the LRR motif between them. Suggested LRR-structure containing templates available in PDB are 5HYX, 5GIJ, 5IXT, 5B0T, and 4Z5W. All of these PDB files contain a signature "horseshoe" shape with a parallel beta sheet inside the curved structure. These templates are loaded into the program by clicking the "Query PDB" option in the "File" tab followed by entering the protein IDs. Viewing options similar to the above section are recommended for 3D visualization of the protein structures. Again, students experience guided-inquiry learning as they complete questions in the exercise sheet by using the information obtained (Supporting Information Material, Exercise A, Q2).

Pre-Experimental Exercise 2: DNA Sequences Reverse Complement and Translation Exercise

In this section, information on two degenerate primers used in the next part of the laboratory exercise is given. The primers are LM-638 (5′-GGIGGIGTIGGIAAIACIAC-3′)

TABLE 1 *Materials and amount needed for each reaction*

Material	*Required amount per group*	*Expected amount for 10 groups*
TRIsure™ reagent	800 μL	10 mL
Chloroform	200 μL	2.5 mL
Isopropyl alcohol	200 μL	2.5 mL
SensiFAST™ One-Step Real-Time PCR kits	1 reaction	15 reactions
Agarose powder	1.5 g	3.0 g
1× TAE buffer	300 mL	600 mL
Gene Ruler™ 1 kB DNA ladder	2 μL	4 μL
5× DNA loading buffer blue	2 μL	4 μL
Midori Green stain	0.1 μL	0.2 μL

and LM-637 (5′-ARIGCTARIGGIARICC-3′), chosen for use as they have been robustly tested in other previous studies and reported to be operational in many different plants [18]. The forward primer LM-638 was designed based on the P-loop [GGVGKTT] motif, and the reverse primer LM-637 was designed based on the hydrophobic [GLPLAL] motif region [19]. Students then attempted the translation of the degenerate primer sequences into their respective amino acids sequence, with the aim of predicting the targeted amino acids sequence of both the NBS and LRR motifs (Supporting Information Material, Exercise B). During this session, a revision (discussion) lecture is included for the instructor to go through relevant theory and background information to ensure students possess sufficient understanding prior to the beginning of the practical (wet-lab) sessions. Example topics comprised of nucleic acid extraction methods, the basic principle of PCR, primer design (length, GC content, T_m of primer set), and other information such as mispriming, primer dimer formation, and issues of smearing due to poorly designed primers.

Wet-Lab Experiment: PCR Amplification of Target Amino Acid Sequences Using Degenerate Primers

Flowchart and Time Table of the Wet-Lab Experiment

All materials required for this experiment are listed in Table 1, whilst an estimation of the time taken for each of the major steps of the experiment is summarized in Table 2.

Total RNA Extraction

Fresh young leaves of *P. colubrinum* are preferred, but leaf samples collected and kept frozen at −80°C can also be used. Total ribonucleic acid (RNA) was extracted using the TRIsure™ reagent (Bioline, MA, USA) following the manufacturer's instructions. Briefly, 100 mg of leaf tissue was pulverized in liquid nitrogen into a fine powder and placed into a 1.5 mL microcentrifuge tube. Then 800 μL of TRIsure™ reagent is added to the sample and vortexed for 1 min. The mixture was left at room temperature for 2 min, after which 200 μL of chloroform was added, mixed well and the sample centrifuged at 12,000 rpm for 5 min. The supernatant was carefully transferred to a new 1.5 mL microcentrifuge tube and 400 μL of chilled isopropyl alcohol was added. The tube was placed into a freezer at −20°C for 20 min for the precipitation of RNA molecule. Next, the sample was centrifuged at 12,000 rpm for 20 min and the

TABLE 2 *Steps and estimated time needed for the experiments*

Steps	*Estimated time needed for 10 groups (min)*
RNA extraction	50
RT-PCR preparation	20
Incubation of RT-PCR in thermocycler	50
Preparation of agarose gel	
Gel electrophoresis	30
Visualization of PCR products	10
Gel documentation	5

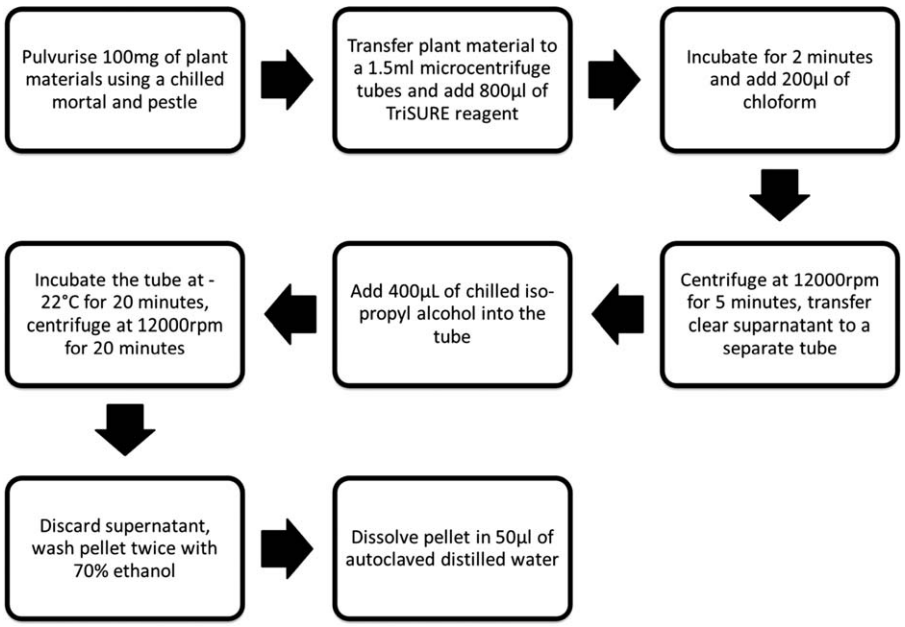

FIG 2 *Process flowchart for RNA extraction method using TRIsure™ reagent.*

supernatant discarded. The visible total RNA pellet was washed twice with 1 mL of 70% ethanol. The final total RNA pellet was air-dried in room temperature (RT) for 20 min and then dissolved with 50 µL of autoclaved RNase-free water and stored immediately at −80°C until further use. Typically, extraction of one sample will generate an adequate amount of total RNA for 45 Reverse Transcription-Polymerase Chain Reaction (RT-PCR) reactions. A simplified summary of the protocol is depicted in Figure 2.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

One microliter of total RNA extract was then used for reverse transcription and polymerase chain reaction. SensiFAST™ One-Step Real-Time PCR kit was obtained from Bioline. In the One-Step RT-PCR reaction, RNA is converted to cDNA and serves as the template for the PCR. Following the manufacturer's instructions, the recipe of one reaction is as follows: 12.5 µL of 2× reaction mixes, 1 µL of template RNA, 1 µL of forward primer, 1 µL of reverse primer, 0.25 µL of polymerase, 0.4 µL of RiboSafe RNase Inhibitor, added with 8.85 µL of autoclaved distilled water. The heating cycles of the reaction were as follows: cDNA synthesis 1 cycle: 50°C for 15 min; denaturation 1 cycle: 94°C for 2 min; PCR amplification 40 cycles: 94°C for 15 s (denaturation), 55°C for 15 s (annealing), 72°C for 15 s (extension) and final extension 1 cycle: 68°C for 5 min. Negative controls were prepared by substituting RNA template with autoclaved distilled water.

Agarose Gel Electrophoresis and Visualization of PCR Products

The PCR products were assessed by gel electrophoresis on a 1.5% agarose gel (prestained with 2 µL/L Midori Green) together with a DNA ladder (Gene Ruler 1 kb DNA ladder) and negative controls. Two microliters of 5× DNA Loading Buffer Blue was added to 8 µL of PCR products and mixed thoroughly by pipetting. Each sample was loaded carefully into each of the wells of a submerged agarose gel. Electrophoresis was performed at 100 V until the loading dye reached 70% of the total gel length. The results were documented with a gel documentation system (or ultraviolet transilluminator).

Expected PCR Results on Gel Electrophoresis

In this exercise, due to the presence of multiple resistance genes in plant genomes, the NBS–LRR targeted amplification of DNA was expected to yield a heterogeneous mixture of fragments that migrated in an agarose gel as a single band of approximately 500 bp in length. Figure 3 shows a typical result of the PCR products. The Gene Ruler 1 kb DNA ladder (Lane 1) served as a molecular DNA size marker. Lanes 2–4 and lanes 9–10 showed PCR products from three different RT-PCR reactions while lanes 5–7 and lanes 10–13 are negative controls. A positive result is indicated by a thick, bright band of approximately 500 bp in size while the negative controls do not have any band (no amplification).

Safety Issues and Precautions

For the total RNA extraction and RT-PCR steps, all glassware, plastic consumables and reagents used have to be RNase-free. It is also advisable to preclean the laboratory work areas and handling pipettes with RNase cleaning agent

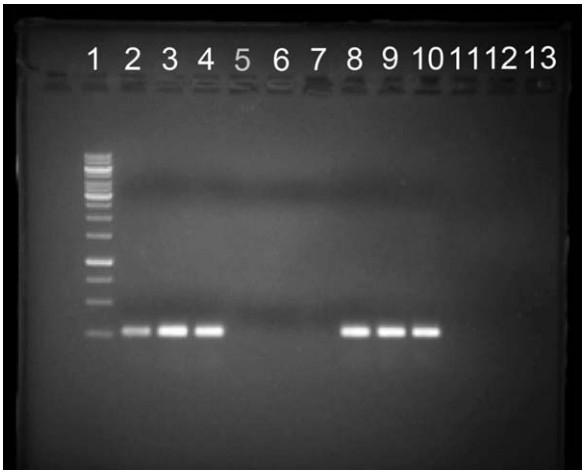

FIG 3 *Gel image of a representative 1.5% agarose gel used to examine the PCR products. Lane 1: GeneRuler 1 kb DNA ladder as a molecular DNA molecular weight marker; lanes 2–4: Typical expected PCR products; lanes 5–7: negative controls; lanes 8–10: typical expected PCR products; lanes 11–13: negative controls.*

to minimize RNase contamination. Standard personal protective equipment (PPE), which includes the use of gloves, lab coats, long trousers, and covered shoes, should be enforced during the laboratory session. Main safety risks in this experiment involve the handling and use of liquid nitrogen, exposure to hazardous chemicals (namely TRIsure™ reagent and chloroform), and handling a high voltage power pack during gel electrophoresis. All used materials are properly disposed of as hazardous waste after the experiment.

Discussion

The wet-lab part of this laboratory exercise includes several well-established molecular techniques: total RNA extraction from a wild species of black pepper, amplification of partial gene sequences using cDNA template and RT-PCR, and visualization of PCR amplicons using agarose gel electrophoresis. The primers were initially designed for use in soybean and had also been found to work in several different plants such as rice, grapevine, and wheat seedlings [20, 21]. Therefore, this approach should not only be applicable to isolate RGAs in *P. colubrinum* but also adaptable to other crops if pepper plants are not available.

Occasionally, some groups might fail to obtain results due to one or interplay of several reasons. Three common observations of an unsuccessful PCR experiment are the absence of bands of the correct size, the presence of nonspecific bands, or smeared lanes. An absence of band indicates a failed PCR reaction due to the presence of inhibitors or nonoptimized PCR conditions. Some inhibitors such as ionic detergents, phenol, heparin, xylene, cyanol, and bromophenol blue can interfere with the PCR reaction and result in no PCR product [22]. The problematic template may be diluted by 100 to 1,000-folds to reduce the concentration of inhibitors prior the rerun of PCR, however in many cases, re-extracting the total RNA might be necessary to resolve this issue.

The presence of multiple or nonspecific bands (including primer dimer) might indicate nonoptimal melting/annealing temperature in the heating cycles. Temperatures used in the PCR cycles can be improved by running a gradient PCR or using hot-start or touchdown PCRs. Band smearing indicates pervasive nonspecific primer binding or the overabundance of the cDNA template. Concentrations of chemical components such as dNTP, $MgCl_2$, or primers in the reaction mix then need to be optimized to increase stringency during the annealing phase. Further details on optimization and troubleshooting of PCR can be found in a review prepared by Roux [22].

Lastly, this laboratory exercise is amenable to suit a broader range of educational context. A research/discovery component can be emphasized by expanding and continuing the experiment with molecular cloning and DNA sequencing. In such situation, a PCR gel extraction/purification kit is needed to isolate the PCR bands. Purified PCR extracts can then be directly cloned into plasmid vectors with the use of commercial kits (many PCR-based cloning kits are available). Any number of positive colonies can be selected, cultured and subjected to plasmid DNA extraction. Plasmid DNAs can then be sent out for DNA sequencing. The sequences obtained can be further characterized using in silico analysis such as BLAST, PFAM to determine individual RGAs (potential resistance genes) that exist in the plant species. Candidate genes can also be targeted for full gene sequence isolation. The methodology and detailed protocols for these downstream techniques have been previously reported [6].

Educational Implication

This paper describes a laboratory exercise designed to aid undergraduate students to correlate the concepts of protein structures and functions by integrating molecular techniques, database and bioinformatics software to analyse protein motifs of plant resistance genes. A real-world crop example (black pepper and its wild relative *P. colubrinum*) and plant-pathogen based protein interaction were used in the laboratory exercise so that students are able to relate the knowledge gained with its actual application in research and the crop (pepper) industry. This laboratory exercise has components of authentic and discovery learning styles [23, 24]. A blended learning method is achieved in this exercise by introducing students to the basics of DNA sequence translation, multiple sequence alignment of amino acid sequences, and 3D protein structure visualization process using online database information and bioinformatics software. This not only strengthens the student's understanding of protein motifs-structure but also exposes them to some basics skill sets required for the study of

computational biology. A second part is followed with hand-on experiments using various molecular techniques such as total RNA extraction, application of degenerate primers for amplification of partial gene using RT-PCR, and visualization of DNA fragments using gel electrophoresis.

Student Feedback

Anonymous feedbacks were documented from 12 final year undergraduate students from the Biotechnology Program at the end of the laboratory exercise. Overall student feedback has been positive, and a shared positive comment expressed interest and liking for the bioinformatics tools (e.g. "The module is quite interesting, playing around with all the software enhance understanding."). However, a few students found the exercise challenging since it was their first time using the software (e.g. "First time dealing with the software and not familiar with the interface, instruction with an attached image of the interface may help me to understand more"). From the feedback, it was found that having the amino acid alignment and protein modelling exercise prior to the wet-lab increased interest of the students towards the experiment.

In conclusion, this laboratory exercise is intended to introduce and connect the concepts of protein secondary structures and function and targeted gene amplification from cDNA with degenerative primers using RT-PCR technique. Feedback comments showed that the students displayed a high level of interest and enthusiasm, especially towards the bioinformatics exercise given.

Suppliers

TRIsure™ reagent (Cat no: BIO-38032), GeneRuler 1 kb DNA Ladder (Cat no: BIO-33053), 5× DNA loading buffer blue (Cat no: BIO-37045), and SensiFAST™ One-Step Real-Time PCR kits (Cat no: BIO-72001) were obtained from Bioline, USA (http://www.bioline.com/us/). Chloroform (Cat no: 288306), isopropyl alcohol (Cat no: I9516), TAE buffer (Cat no: T8280), and agarose powder (Cat no: A9539) were obtained from Sigma-Aldrich (http://www.sigmaaldrich.com). Lastly, Midori green solution (Cat no: MG04) was obtained from Nippon Genetics Europe (https://www.nippongenetics.eu).

Disclosure

The authors declare no conflict of interest.

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