Differential Display Reverse Transcription Polymerase Chain Reaction (DDRT-PCR) for Grey Oyster Mushroom Samples Grown with Acoustic Sound Treatment

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Abstract. Grey oyster mushroom is the most often farmed and marketed type of mushroom for household consumption in Malaysia. The mushroom cultivation takes a long time due to its complicated mycelium growth. Several treatments were introduced such as acoustic sound treatment to increase the growth rate and quality of the crop, but there is no in-depth study regarding the genetic expression of the genes affected. This study aims to evaluate the Differential Display Reverse Transcriptase-Polymerase Chain Reaction (DDRT-PCR) for grey oyster mushroom samples grown with acoustic sound treatment. The mushroom was subjected to the treatment and the RNA was extracted from the mushroom samples and converted into cDNA before undergoing DDRT-PCR. Ten Differential Expressed Transcripts (DETs) that were successfully identified based on the differences between the intensity and absence of amplicons were sent for gene sequencing and BLAST through the NCBI database to obtain relevant results regarding the possible gene annotation. Seven out of ten DETs hit potential genes encoding for housekeeping and structural and development functions. The results showed that acoustic sound treatment did affect the expression of certain genes differently as captured by DDRT-PCR analyses and offers new ideas for the development of ecological agriculture.

1. Introduction

Grey oyster mushroom, also known as *Pleurotus sajor-caju*, is now grown around the globe mainly for commercial purposes. The growth and quality of the mushrooms play an important factor in the commercial production line. Therefore, several physical treatments have been researched and performed to evaluate their effects to the production of grey oyster mushroom such as heat shock, temperature, light intensity and acoustic sound treatment [1]. Sound is an oscillatory concussive pressure wave transmitted through gas, liquid, and solid. Previously treatments using different acoustic sound at 75 dB has been studied and considered as a better treatment to enhance the mycelium growth and thus accelerate the mushroom cultivation process to increase the mushroom productivity [2]. The treatment implicated on mushroom during its growth and development is expected to also leads to an alteration of its gene expression. Gene expression is the process through which information from a gene is utilized in manufacturing functioning gene products such as RNA and proteins [3].

Whether in a simple unicellular organism of bacteria or a complex multicellular organism such as fungi, plants and animal, each cell controls when and how its genes are expressed which allows the cell to adapt to different conditions. Differential display reverse transcription-Polymerase Chain Reaction (DDRT-PCR) is a technique that has been proven to be highly effective in identifying sequences that are differentially expressed in various cell types. This approach is a laboratory technique that allows a researcher to compare and identify changes in gene expression at the mRNA level between two or more eukaryotic cell samples. The technique of Differential Display Reverse Transcription PCR (DDRT-PCR) has been used to evaluate differentially expressed transcripts in several different growth conditions using numbers of fungi or mushroom species including *Letinus edodes* (shiitake) [4], *Morchella conica* [5] and *Piptoporus betulinus* [6]. Currently, there is no report related with the evaluation of the differential expressed transcripts (DETs) of the grey oyster mushroom samples treated with acoustic sound by using DDRT-PCR has been published yet. Therefore, this study aims to evaluate the DDRT-PCR analyses for grey oyster mushroom samples grown with acoustic sound treatment.

2. Methods

2.1. Mushroom sample preparation and total RNA extraction

Mycelium samples (19th day after spawning stage) were used and the treated sample undergone two sessions of acoustic treatment. The samples were categorized into two, which are treatment and control sample. Treatment sample was treated with acoustic sound while control sample did not treated with acoustic sound. The treatment samples were exposed towards acoustic sound with 15 pops of firecracker sound (75 decibels) every 10 days until fruiting bodies appeared after 5 days of inoculation. Both samples were grown using the same substrate components, inoculation, incubation and storage condition as described by Anjum et al. [2] with slight modification. Total RNA extraction step was performed according to VIVANTIS GF-1 Total Extraction Kit protocol with slight modification. Approximately 70 g samples of the treated and control mushrooms were used with the addition of 10 µl of Proteinase K.

The samples were vortexed briefly before being transferred into an incubator at 65 °C for 20 minutes at the homogenization stage (shaking the tube invertedly every 5 minutes). An ice box was used to prevent degeneration of RNA due to temperature. Approximately 30 µl of RNase-free water was added directly onto the membrane at the isolation step and allowed to stand for 5 minutes before being centrifuged at 10,000 x g for 1 minute (repeated 3 times). The extracted RNA was stored at -20 °C (short-term storage) or -80 °C (long-term storage). The extracted RNA samples were then visualized by 1% agarose gel electrophoresis with the addition of bleach at 90 V power for 45 minutes. The Gel Documentation system was used to visualize the RNA band's result.

2.2. Isolation of mRNA, cDNA synthesis and validation with specific target region primers

The mRNA magnetic isolation module (NEB) was used to isolate the messenger RNA according the manufacturer. The process was conducted on ice to keep the reaction mixture in the optimum condition. The eluted mRNA of the treatment and control samples were reverse transcribed to cDNA by First Strand cDNA synthesis kit (NEB) with anchored primer T12A (Table 1). The mixture was incubated in an incubator at 42 °C for one hour, then the enzyme was inactivated at 80 °C for 5 minutes in the thermal cycler machine. The cDNA products were stored in the freezer at temperature of -20 °C.

The cDNA synthesized from mRNA was amplified by using Polymerase Chain Reaction (PCR) with a universal primer pair which are 18S-NS1/NS2 (Table 1) as positive control as well as to validate the quality of the samples. PCR was carried out in a final reaction volume of 50 µl containing 60ng/µl of first strand cDNA, 25 µl of 2X Master Mix (Lucigen), 1µM 18S-NS1/NS2 primers and water up to total 50 µl. Thermal cycler was preheated to 94 °C before being operated. The amplification of samples was conducted for 30 cycles after initial denaturation at 94 °C for 30s, elongation at 55°C for 30s, 72°C for 45s and final extension at 72°C for 5 min. The amplified products were visualized on 1% agarose gel and stained with ethidium bromide by gel documentation system.

2.3. DDRT-PCR, DNA Sequencing and in silico analysis

PCR amplification of each cDNA (treatment and control) was carried out in nine combination includes one of the nine arbitrary primers [7, 8] with an anchored primer DD12 (Table 1) as described by Iqbal et al. [9] with slight modification. The amplification was carried out in a final reaction volume of 50 µl containing 50 ng/µl of first strand cDNA, 25 µl of 2x Master Mix (Lucigen), 2 µl of anchored primer (250 ng/µl), 8 µl of arbitrary primer (100 ng/µl) and water up to total volume of 50 µl. The protocol for DDRT-PCR was as follow; first cycle at 94°C for 4 min, 36°C for 2 min, and 72°C for 2 min, followed by 39 cycles at 94°C for 1 min, 36°C for 1 min, 72°C for 1 min and a final extension step at 72°C for 10 min. The process was repeated with another combination of anchored and arbitrary primers as shown in Table 2.

The amplified cDNA was visualized by 1% agarose gel electrophoresis and stained with ethidium bromide and viewed using gel documentation system. The bands were examined and the size of amplified cDNA was determined by comparing the product size with ladder size. The differentially expressed bands were excised from the gel and sent for gel elution single pass DNA sequencing by local service provider. The acquired cDNA sequences were compared to the sequences in GenBank. The Basic Local Alignment Search Tool (BLAST) was used to search the cDNA database of the National Center for Biotechnology Information (NCBI) at (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 1. Primers, sequences and their applications.

Application	Primer Name	Primer Sequence (5'-3')
Validation of cDNA	18S-NS1	GTAGTCATATGCTTGTCTC
Validation of cDNA	18S-NS2	TCCTCCGCTTATTGATATGC
DDRT-PCR (Anchored Primer)	DD12	TTTTTTTTTTA
DDRT-PCR (Arbitrary Primer)	A-03	AGTCAGCCAC
DDRT-PCR (Arbitrary Primer)	A-09	GGGTAACGCC
DDRT-PCR (Arbitrary Primer)	A-16	TTTTTTTTTTA
DDRT-PCR (RAPD Primer)	RAPD 6	GATCCCCTGA
DDRT-PCR (RAPD Primer)	RAPD 12	TAACCATCCC
DDRT-PCR (RAPD Primer)	RAPD 18	GAAACGGGTG
DDRT-PCR (RAPD Primer)	RAPD 24	AGGTGACCGT
DDRT-PCR (CDDP Primer)	CDDP – WRKY F1	ATGAATTCTTTTACTAGCAA
DDRT-PCR (CDDP Primer)	CDDP – WRKY R1	TCAGTTAAGGAAAGAGC

3. Results and Discussion

3.1. Analysis of total RNA and validation of the synthesized target cDNA samples

According to Figure 1(A), there are two bright bands at approximately 2500 bp and 1500 bp in size were observed as a result for gel electrophoresis. The extracted total RNA from mycelium of the samples showed clear 28S and 18S ribosomal RNA bands on agarose gels. The clear bands indicated that the extracted total DNA is suitable for subsequent downstream analysis and not degraded. Extraction of total RNA from mycelia of a dimorphic fungal species as well as White Jelly Mushroom also showed two clear and significant bands of 28S and 18S ribosomal RNA [10, 11]. Furthermore, with the help of a molecular reader, both concentration of the RNA is approximately 340 ug/nl and an absorbance ratio A260/ A280 of approximately 2.0 which can be considered to be of high purity and less salts, hence it can be concluded that the total RNA was successfully isolated from both grey oyster mushroom samples. Therefore, the total RNA isolated from the samples are pure, integrated and stable for cDNA synthesis.

RNA obtained must be directly converted into cDNA due to its sensitive nature, which if not stored properly under controlled environment, will degenerate quickly. mRNA was isolated from RNA before

being converted to cDNA. The presence of mRNA and its detection is the key point to find out about gene expression and synthesis of proteins from specific genes because not all the genes are transcribed all the time in all cells and developmental stages [12]. Therefore, mRNA was isolated to ensure the accuracy and purity of the cDNA acquired later on. The use of mRNA in cDNA synthesis will reduce the number of low molecular weight amplification products, thus yielding fewer bands on the gel and lower chances of getting heterogeneous population of polymerase chain reaction products of same size [9]. The differentially expressed genes are easily identified and may be eluted from the gel easily.

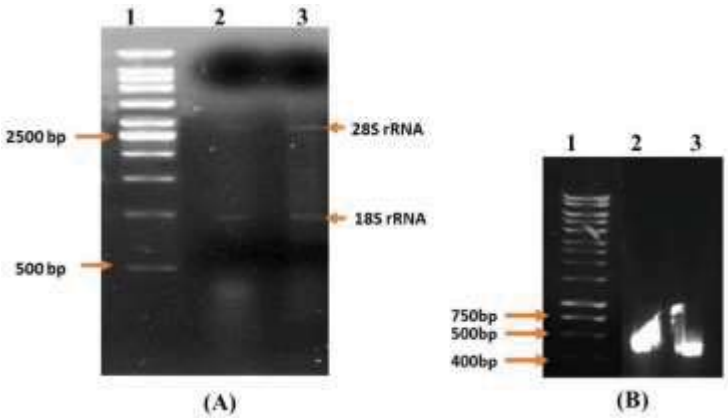

Fig. 1. (A) Genomic RNA extracted from grey oyster mushroom resolved in 1% (w/v) agarose gel. (Lane 1: 1kb Vivantis DNA Marker, Lane 2: treatment, Lane 3: control; **(B)** cDNA validation after PCR amplification with NS primers resolved in a 1 % (w/v) agarose gel at 56 °C annealing temperature (Lane 1: 1 kb Bioron DNA Marker, Lane 2: treated, Lane 3: control).

mRNA was used in the cDNA synthesis with an anchored primer, namely DD12. A total of 20 µl of final cDNA was obtained per sample through incubation of 42 °C for an hour before deactivating the enzymes at 80 °C. It is important to stop the reaction of the enzymes to prevent further unwanted reactions to occur. An extra optimization method can be utilized in the future which is pre-heating and snap chilling the primers which in return will melt any secondary structure formed due to the repetitive dT sequences and prevent the secondary structure from reforming [13]. The PCR amplification of cDNA validation was successfully carried out by using primer 18S-NS1/NS2 at an optimized annealing temperature of 56 °C. Figure 1(B) shows that the bands produced at the estimated size in between 400 bp and 500 bp. Therefore, the cDNA obtained in this study were considered high quality and intact. The same target sequences previously tested for samples *C. acutatum* and *C. gloeosporioides* with the production of bands at size between 500-600 bp [14].

3.2. Amplification of cDNA using DDRT-PCR, gel electrophoresis and DNA sequencing

PCR amplification of each cDNA was carried out in combination with one of the nine arbitrary primers with the anchored primer resulting in nine combinations. A different first cycle protocol condition was performed in order to properly denature the sample before running. During the annealing phase of PCR, the reaction temperature needs to be sufficiently low to allow both forward and reverse primers to bind to the template, but not too low as to enable the formation of undesired, non-specific duplexes or intramolecular hairpins, both of which reduce reaction efficiency. The annealing temperatures for different types of arbitrary primers were different and were selected in accordance to the Tm of the primers and master mix. 50 °C annealing temperature was used for CDDP primers and 36 °C was used for A0 and RAPD primers. The results were shown in Figure 2A, Figure 2B and Figure 2C.

From the figures, there were few significant differences between treatment bands and control bands based on the intensities and absence, which had been categorized into differentially expressed transcripts (DETs). Ten DETs were identified in total throughout the study. DET 1, 6, 10 were selected due to the presence of extra band in contrary with the other samples. The rest of the DETs were selected based on

the differences in the intensity of the bands produced. The bands obtained from CDDP primers have no significant differences in intensity between treatment and control, therefore it is excluded for the subsequent analyses. The details of the DETs determined are shown in Table 2. The DETs determined from treatment samples namely DET 4, 5, 7 and 8 contained brighter bands and DET 1 and 9 contained extra bands in comparison to the control samples. It can be postulated that the differences in genetic expression are due to the exposure to the acoustic treatment which is considered as a type of stress during growth and development. The differentially expressed transcripts in the result are in respond to such stress implicated by the treatment.

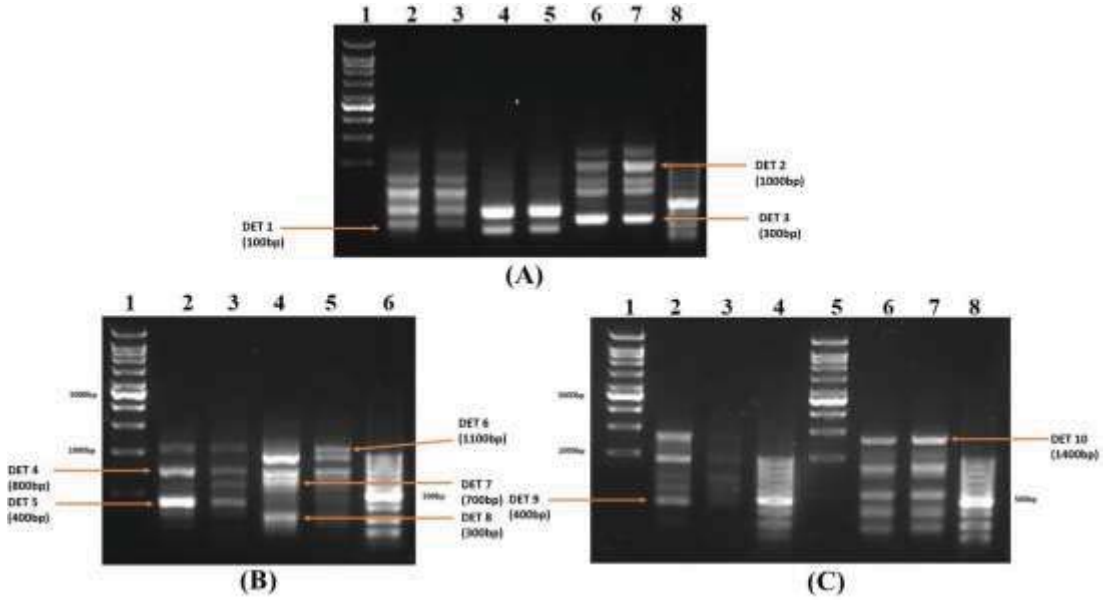

Fig. 2 Electrophoresis result of DDRT-PCR product : **(A)** 6 samples (Lane 1: 1 kb Vivantis DNA Ladder, Lane 2: Treatment with A03, Lane 3: Control with A03, Lane 4: Treatment with A09, Lane 5: Control with A09, Lane 6: Treatment with A16, Lane 7: Control with A16, Lane 8: 100 bp Vivantis DNA Ladder); **(B)** 4 samples (Lane 1&5: 1 kb Vivantis DNA Ladder, Lane 2: Treatment with RAPD 18, Lane 3: Control with RAPD 18, Lane 6: Treatment with RAPD 24, Lane 7: Control with RAPD 24, Lane 4&8: 100 bp Vivantis DNA Ladder); **(C)** 6 samples (Lane 1: 1 kb Vivantis DNA Ladder, Lane 2: Treatment with A03, Lane 3: Control with A03, Lane 4: Treatment with A09, Lane 5: Control with A09, Lane 6: Treatment with A16, Lane 7: Control with A16, Lane 8: 100 bp DNA Ladder).

Table 2. Differentially Expressed Transcripts (DETs).

DET	Primer	Type of Sample	Size (Bp)	Reason of Selection
1	A03	Treatment	100	Contained extra band
2	A16	Control	1000	Brighter band
3	A16	Control	300	Brighter band
4	RAPD 6	Treatment	800	Brighter band
5	RAPD 6	Treatment	400	Brighter band
6	RAPD 12	Control	1100	Contained extra band
7	RAPD 12	Treatment	700	Brighter band
8	RAPD 12	Treatment	300	Brighter band
9	RAPD 18	Treatment	400	Contained extra band
10	RAPD 24	Control	1400	Brighter band

Four DETs from control samples namely DET 2, 3 and 10 produced brighter bands and DET 6 produced an extra band in comparison to the treated samples. The production of brighter bands in the control samples indicate that the gene expression is down regulated in treated samples when exposed to acoustic sound. The down regulated of these few genes and expression of unique gene may correspond to a general response for adaptation to new environment condition when stress was applied. In biological system, one of the examples of basic and important response is translational of mRNA to proteins in protein synthesis that are carefully regulated and changing substantially under different conditions and cell types such as development of functional protein associated with structural and development of mushroom mycelium and fruiting body. Mazidi et al. [15] reported that the acoustic treatment can provide a higher growth rate of mycelium as well as shorter time taken to complete all growth stages.

Yee et al. [16] reported that the role of sound treatment is associated with up regulation of the expression of several processes such as metabolism-related genes related to glycolysis, cell wall biosynthesis, oxidative phosphorylation, and pentose phosphate pathways in the selected plant. Gene sequencing is important in determining the nucleotide sequence for molecular identification study. Depending on conditions and system to be studied, nucleotide sequences in DNA and RNA provide guideline in the cell regulation in an organism, and indirectly make up the fundamental knowledge of the genome and what is happening at that particular condition or system. FASTA format and chromatogram from the sequences were obtained as a result of gene sequencing.

3.3. In silico analysis of DETs and group of potential genes

Sequence analysis of the ten DETs demonstrated that seven of them are encoding for genes that have been previously characterized, while the other three of the DETs obtained less favorable result or "No Search Result" (NSR) from the NCBI database. The 10 DETs were categorized into three potential groups based on their functions and are discussed below (Table 3). DET 5 and DET 8 are encoding genes that are up regulated and DET 1 and DET 9 are producing newly expressed genes in treatment samples as response to the exposure of acoustic sound during their growth. These DET 1, DET 5, DET 8 and DET 9 are housekeeping genes and are responsible for the conversion of genetic messages to functional cell components via the translation of mRNA to proteins. Housekeeping genes in grey oyster mushroom are examples of genomic areas that are highly conserved and evolve more slowly than other genes, such as tissue-specific genes, owing to their responsibilities in the maintenance of basic cellular activities and are required for a cell's survival [17]. The transcript level of the house keeping genes may vary during mycelium development due to the differences in metabolic activities being carried out with mycelium development. Through this, protein synthesis is initiated. Ribosomal RNA is a component of all self-replicating systems; it is readily isolated; and its sequence changes slowly with time according to the cell needs [18]. DET 1, DET 5, DET 8 and DET 9 *in silico* analysis hit with housekeeping genes reported in other organism. The 16S ribosomal subunit of the 30S ribosomal subunit is necessary for the commencement of protein synthesis and the stabilization of proper codon-anticodon pairing at the ribosome's A site during mRNA translation [19].

Table 3. Groups for the Differentially Expressed Transcripts (DETs).

Group	Category of Potential Gene	Number of DETs	DETs
1	Housekeeping	4	DET1, DET5, DET8 and DET9
2	Structural and Development	3	DET7, DET2 and DET10
3	Uncharacterized gene	3	DET3, DET4 and DET 6

At various phases of development, different development-specific genes are expected to be expressed and application of external stimuli may give differences in the way they are expressed. The differential display technique used in this study showed one transcript (DET 7) encoding gene involves in structural and development to be differentially expressed over the acoustic sound treatment. In this study, the

expression of this gene was found to be up-regulated in treated sample. The same gene has been reported in the development of fruiting shells of palm oil which is responsible for the formation of certain attributes of the fruiting bodies appearance. According to Singh et al. [20], the gene called shell gene is responsible for transcription factor that control differentiation of the ovule, seed and lignified endocarp. Another two transcripts in this same group of encoding structural and development gene, DET 2 and DET 10 were down-regulated when exposed to the treatment. Both genes were reported to be involved in the development of functional proteins including gene transcription process as well as protein production. The phase during which the initial nucleotides in the RNA chain are produced is known as transcription initiation [21]. Without proper expression of DET 10, the initiation of RNA synthesis may not be halt and protein would not be able to be produced as the primary function of RNA is to create proteins via translation.

Three DETs have been grouped under uncharacterized gene including undefined protein which are DET 3, DET 4 and DET 6 since the result from *in silico* analysis did not represent clear hit with any specific genes. There are one DET (DET 3) which contained codes for undefined protein in NCBI database and two DETs (DET 4 and DET 6) which have no results obtained from blasting. The percentage of identities of the blast search of all DETs are ranging from 68 to 94 percent. Almost all functional prediction methods rely on the identification, characterization, and quantification of sequence similarity between the gene of interest and genes for which functional information is available. The process could be improved by applying subsequent procedure of gene cloning to improve the sequences covered during the reading of gene sequencing as well as the use of additional bioinformatics tools. The function of these potential genes remains to be determined but it is likely that these genes may have some role at the structural growth and metabolism of grey oyster mushroom. Since grey oyster mushroom have not much genomic information available, it could also be deduced as a novelty sequence obtained. This is due to the randomness in DDRT-PCR procedure in detecting specific gene.

4. Conclusion

RNA was successfully extracted from the mycelium of grey oyster mushroom with good quality and concentration. cDNA was successfully converted from mRNA and being used in subsequent analyses. Primer 18S-NS1/NS2 was used to amplify the 18S region of the cDNA at an optimum annealing temperature of 56 °C. Ten DETs were identified from the overall result which contains significant differences between the treated and control samples. The DETs were later successfully cut and sent for Sanger sequencing and BLAST through NCBI database. The results obtained can be categorized into three functional categories of potential genes which are housekeeping genes, structural development related genes and uncharacterized genes. In conclusion, a total of 7 DETs encoding for genes which are possibly affected by the acoustic sound treatment are detected through this study. The results of this study provided molecular insight into how grey oyster mushroom responded to acoustic sound treatment and how certain genes are captured to be differentially expressed by DDRT-PCR over the treatment.

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