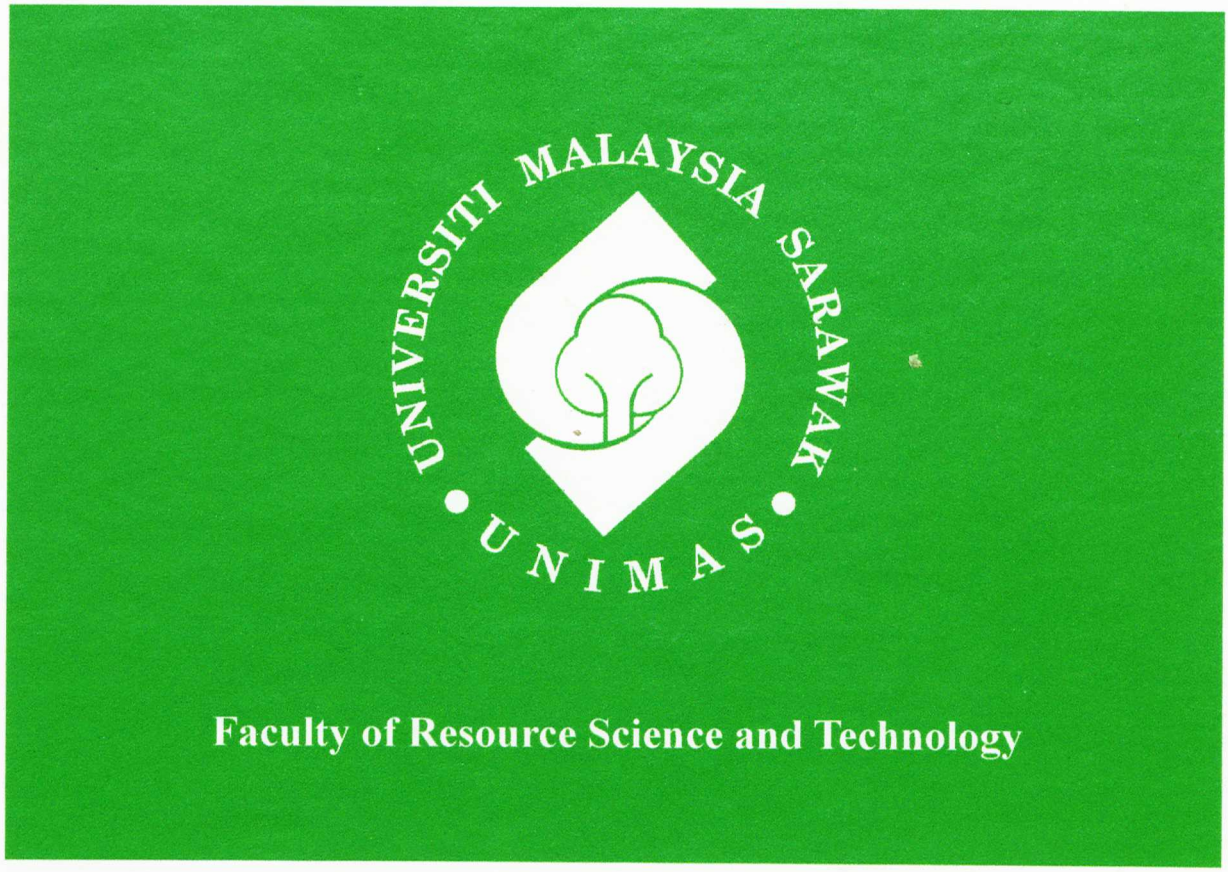

Comparative Proteome of Irradiated Mutant and Wild Type *Oryza Sativa*

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Bachelors of Science with Honours
(Resource Biotechnology)
2018

Comparative Proteome of Irradiated Mutant and Wild Type *Oryza Sativa*

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A project thesis submitted in partial fulfillment of the Final Year Project (STF 3015) Course

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Date: 15/06/2018

ACKNOWLEDGEMENT

My deepest gratitude goes first to my supervisor, AP. Dr. Mohd Hasnain Bin Md Hussain for his technical support and encouraging association throughout the period of my research work in Faculty of Resource Science and Technology, Proteomic lab, UNIMAS.

I would like to express my gratitude to Nikson Fatt Ming Chong for providing the technical guidance and mentorship during the period in which this research work was carried out. Mentoring and encouragement have been especially valuable and his directorial insights launched the greatest part of this dissertation.

I am also gratified to my coworkers which work along in the lab for providing a positive working atmosphere in the laboratory. I also would like to thank my parents for their inspiration and moral support in completion of this research.

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ABSTRACT

Comparative proteome of irradiated mutant and wild type *Oryza sativa*

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In this research, the comparative proteome is the protein analysis of irradiated mutant and wild type *Oryza sativa* to detect the presence or absence of proteins and the direct measurement of relative protein abundances. This is done first by the extraction of total protein from the sample, and then protein was separated by using SDS – PAGE and 2D – PAGE. These proteomic methods were used to obtain a protein profile of rice total seed proteins and identify important functional protein component. Then, results were analyzed using Delta2D – Gel analysis software. Overall, matching protein spots of irradiated mutant varieties of Mutant 28, Mutant 39 and Mutant 40 with variety MR219 – wild type were 101, 103 and 107 respectively. These results expand our understanding of the rice proteome and improve our knowledge of the cellular biology of rice seeds. To obtain the new desirable trait in crops, mutation breeding was vastly used. Since, mutation cause changes in protein expression, protein analysis was done to know which protein undergo alteration.

Keywords: *Oryza sativa*, SDS – PAGE, 2D – PAGE, Delta2D Gel analysis software

ABSTRAK

Dalam kajian ini, perbandingan profil protein di dalam mutan Oryza sativa yang diradiasikan and variasi Oryza sativa yang dominasi melalui perbandingan kehadiran and ketiadaan protein. Pertama, protein diekstrak daripada sampel, kemudian protein akan dikesan melalui permisahan mengunakan SDS PAGE dan 2D PAGE. Cara ini selalu digunakan untuk memperoleh peta rujukan bagi jumlah protein penuh yang terkandung di dalam beras and dengan mengenalpasti molekul penting di dalam profil protein yang diperolehi. Kemudian, keputusan terakhir akan dianalysis menggunakan software analisis 2D Page iaitu . Secara keseluruhannya, protein spot bagi mutan irradiasi dalam variati Mutant 28, Mutant 39 dan Mutant 40 dibandingkan dengan variati MR219 – wild type adalah 101, 103 dan 107 masing - masing. Melalui keputusan ini pengetahuan tentang profil protein beras yang dikaji akan meningkat dan membolehkan kita memahami biologi bagi biji beras. Dengan ini, ciri - ciri yang dikehendaki oleh pengkaji dapat dihasilkan bagi memperoleh baka beras yang lebih bagus.

Keywords: Oryza sativa, SDS – PAGE, 2D – PAGE, Software gel analisis Delta2D

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List of Abbreviations

2D	– Two dimensional
ACN	– Acetonitrile
APS	– Ammonium per sulphate
BSA	– Bovine serum albumin
CAD	– Collisional – activated dissociation
DTT	– Dithiothreitol
EDTA	– Ethylenediaminotetraacetic acid
EGTA	– Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid), also known as egtazic acid, better known as EDTA.
IEF	– Isoelectric focusing
MALDI	– Matrix Assisted Laser Desorption/Ionization
MS	– Mass spectrometry
PAGE	– Polyacrylamide gel electrophoresis
pI	– Isoelectric point
pKa values solution	– Negative base-10 logarithm of the acid dissociation constant (K_a) of a
PMSF	– Phenylmethane sulfonyl fluoride or phenylmethylsulfonyl fluoride
SDS	– Sodium dodecyl sulfate
TEMED	– Tetramethylene – diamine

1. INTRODUCTION

It is important to keep improving the breeding strategies in agriculture as the human population is increasing. Since human population is growing, the natural resources might get insufficient. Hence, hybridization method is applied to many foods and feeds in order to meet the need of everyone (Hodgins, 2013). In brief, many of the foods consumed by people worldwide have been altered genetically. This is for the sake of shortening the time of crop production and acquiring desired genetic trait such as more nutritious and tasty. This desired altered trait is called variation (Mba, 2013). Hence, variation is the main goal in breeding that plant breeder is focus on. Through the studies of the mutation breeding, these variations properties can be used to produce one plant with many desired features. In a process called mutation induction, many genetic variety for crops is induced using radiation. This is an important breakthrough as varieties are an important factor in agriculture (Joint FAO/IAEA Programme, 2017). In mutation breeding, identifying the proteins altered through the mutation is what matter the most. Hence, the functionality of the protein is studied. Since, the knowledge can be used for genetic engineering of the crop.

One of challenges in proteome analysis is to separate complex protein components while maintaining the interactive associations among component protein (Zubarev, 2013). In this research, the proteome study is used to determine which proteins in the irradiated mutant rice were affected by the random mutation caused by the gamma ray. In different states of an organism, the protein profiles can be associated to find proteins that are qualitatively and quantitatively affected by the condition of interest. Hence, superior protein separation resolution and technologies are needed for such profiling in order to identify the

potentially huge numbers of proteins (Wrzesinska et al., 2013). This research is aim to study the alteration of protein profile of mutant *Oryza sativa* treated by irradiation comparing with the wild type variety. Through this research, desired trait of rice can be produced by the manipulation of the responsible protein in order to meet the need of human everyday necessities. The hypothesis of this research is that individual proteins which responsible for the mutation in irradiated mutant *Oryza sativa* can be determined by comparing the presence or absence of a protein spot between wild type and irradiated mutant *Oryza sativa* using 2D – PAGE. The alteration of the protein profile may also due to activity of the protein are suppressed not necessarily totally absent.

The objectives of this research are to extract and obtain total protein of irradiated mutant and wild type *Oryza sativa.* Next is to obtain the 2D- PAGE protein profile of irradiated mutant and wild type *Oryza sativa* and then compare the protein profile of irradiated mutant and wild type *Oryza sativa.*

1. LITERATURE REVIEW

2.1 Oryza Sativa (Rice)

Oryza sativa also known as rice comes from perennial grass genus of *Poaceae* family. *Oryza sativa* is a short day plant which is originated from tropics area (Wu et al., 2013). Rice domesticated in India and then brought to China by 3,000 B.C. Which is now cultivated in warm temperate, semi - tropical and wet tropical area worldwide especially for its cereal grain production. Other than wheat, Triticum species, Rice is the two most important cereal crops world for human consumption. Rice was also used as a model for monocotyledon because of it well – establish databases (Song et al., 2012). There are hundreds of cultivars of different grain colour, shape and size of *Oryza sativa*. There are many categories of rice such as valley rice, summer rice, spring rice and upland rice. Transgenic rice is a common event to improve the quality of the rice. The modification of rice done through genetic engineering producing genetically modified rice. There are a lot of improvement have been done resulting in higher quality of rice varieties in term of adding in more nutrients, weight production, pest resistance, and production. One of the transgenic rice is Golden rice, the varieties of rice which produce pro – vitamin A and beta carotene. This may benefit people with vitamin A deficiency (Potrykus, 2010). This is give advantages to solve vitamin A deficiency problem usually faced by under developing countries but reach the subsistence farmers free of charge and no restriction for the farmer to grow their own crops. However, golden rice is not readily accepted in some country since transgenic crops still not well acknowledged. Hence, in order to meet the desire of people, improvising rice quality by genetic engineering should be avoided hence, irradiation of rice to improve its functionality.

2.2 Irradiation of rice mutant

In the late 1920's, through Lewis John Stadler's studies on maize, wheat and barley demonstrated that radiation can produce genetic variability in plants. His studies have been the base start of effective mutation breeding until now. According to El – Degwy, (2013), gamma have been the most rapid method in enhancing the qualitative and quantitative character of many crops. Gamma ray is a highly energetic ray. When gamma ray hits cells, the DNA composition can change through the generation of reactive oxygen. Hence, mutation is induced, if the cell's own repair mechanism do not repaired the changes in the DNA (Iyama & Wilson, 2013). Although mutation itself is a natural phenomenon however radiation can increase the natural mutation. Plant material is irradiated due to the nature of gamma ray which is electromagnetic and particulate. Optimal dose of radiation should be determined before the mutation breeding, it should be high enough to cause mutation and low enough to allow plant grow (Marcu et al., 2012). In the mutation breeding, the mutations usually remain recessive until the following generations (Joint FAO/IAEA Programme, 2017). In Lukanda's studies, compare to various dose treatment, gamma rays treatment at optimum exposure have resulted in high grain yield and other morpho – agronomic parameters. However, too high exposure of gamma ray result in significantly decreases in plant growth and grain yield (Lukanda et al., 2013).

Basically, the mutation breeding seeds are treated in a gamma cells with a Co60 source. Then, in a process called chronic irradiation, whole plants are irradiated on gamma field. The main source of gamma rays used in biological studies is cobalt – 60 and cesium – 137. Since, Cesium – 137 half – life is much longer than cobalt – 60, it is more preferred. The mutation in the DNA structure such as the aberrations of chromosome, substitutions of base,

deletion of base and alterations of base are the reasons of macroscopic variations in phenotypes level. Changes in agronomic traits can be transmitted to the succeeding generations. The aim of shorten the growth time and improve the traits of the crops is achieve by inducing mutations with the combination of different techniques. Compare to the traditional breeding strategies, induced mutations breeding is more effective and time – saving (Hartung & Schiemann, 2014). Besides induced mutations breeding can help to improve quantitative and qualitative traits in crops.

Mutant produced by ion – beam irradiation are not transgenic plants, since transgenic crop have been controversial, the mutants produced by ion – beam irradiation are likely to be accepted by customers (Ishikawa, 2012).

2.3 The Bradford Method for protein quantitation

Calculation of protein concentration is important in the protein analysis procedure. An assay described by Bradford for protein quantitation has been the most chosen method. The Bradford assay depends to the binding of the dye Coomassie Blue G250 to protein. Free dye can exist in four different ionic forms which are the cationic red and green form and the anionic blue form. The anionic blue form binds to the protein and has the absorbance at 590 nm. The concentration of the protein can be determined by the quantity of the dye in the blue ionic form by measuring the absorbance of the solution at 595 nm. However, the dye most willingly binds to arginyl and lysyl residues of proteins which may lead to variation to different proteins. There are two type of assay which are the standard assay and the microassay, standard assay is suitable for measuring between 10 and 100 μg of protein, and

the microassay, can detects between 1 and 10 μg of protein (Walker, 2002). Bradford method is very easy to use, fast and less interference by other substances. However, the contrasting of basic residues between the standard and target protein is not really concerned. Hence, when the standard protein is used, the result may difference. Hence, the standard and target proteins must be the same of accuracy of protein quantitation. It is must better to use standard protein with the same basic amino acid residue ratios to the target protein (Ku et al., 2013).

2.4 SDS – PAGE

One of the most used laboratory method to separated protein is the polyacrylamide gel electrophoresis. One of polyacrylamide gel electrophoresis is Sodium dodecyl sulfate Polyacrylamide gel electrophoresis (SDS – PAGE). SDS – PAGE is used to linearize protein and make protein negatively charged. SDS will bind to the polypeptide chain and cause an equal distribution of charge per unit mass. Negatively charged proteins will move towards the positive electrode throughout the electrophoresis process. The proteins will be separated by size. This electrophoresis use different concentration of two gel systems. The two gels have different electrophoresis buffer composition and pH of electrophoresis tank. There are two layer of electrophoresis gel which are stacking gel for upper layer and separating gel for the lower layer. Stacking gel is a macroporous gel with low concentration (Creative Biomart, 2015). Acrylamide is the material of choice for preparing electrophoretic gels to separate proteins by size. Acrylamide mixed with bisacrylamide forms a crosslinked polymer network when the polymerizing agent, ammonium persulfate (APS), is added. TEMED (N,N,N,N'-tetramethylenediamine) catalyzes the polymerization reaction by promoting the production of

free radicals by APS. Banding pattern of proteins obtained by SDS – PAGE have succeed in identification of varieties in several crop plants (Khan, 2013).

2.5 2D – PAGE

Based on Wittmann et al., (2007), Two – dimensional polyacrylamide gel electrophoresis (2D – PAGE) is able to sort up to 10, 000 protein species from large sets of complex protein mixtures in a single run. As of its name, this electrophoresis have first dimension and two dimension protein separation. Hence it is done vertical and horizontally practice. In the first dimension, proteins are separated based on differences in isoelectric point (pI) value using isoelectric focusing. Isoelectric focusing (IEF) separates proteins by their net charge rather than molecular weight. IEF gels are cast with ampholytes, amphoteric molecules that generate a pH gradient across the gels. Proteins migrate to their pI, the pH at which the protein has no net charge. Since IEF gels contain no denaturing agents, IEF is performed under native conditions (Hegyi et al., 2013).

In the second dimension, they are separated by the molecular weight. Following separation, staining of 2 – D electrophoresis gel is done of protein visualization and analysis. This techniques help to catalog proteins and compare the data among researchers. 2D- PAGE is the combine of two electrophoresis methods which are the SDS – PAGE and isoelectric focusing. Hence, this technique is very effective since the combined consecutive separation steps rely on absolutely independent physicochemical properties. Since, separation based on single property will separated some components but others will remain in the mixture. In both primary and figurative sense, the sample is separated in one dimension. The

components are separated along a single line in the primary sense, while separation is according to pI value in the figurative sense (Hegyi et al., 2013)

2. RESEARCH METHODOLOGY

3.1 Plant materials

Rice (*Oryza sativa*) of variety MR219 – wild type and irradiated mutant caused by ion beam irradiation – 40 and 70 Gy obtained from Nuklear Malaysia, Bangi.

3.2 Extraction and precipitation of total protein from rice grain

In this study, there were three different methods of total protein extraction from rice grain were carried out to compare and obtain the best method for the best to do second dimensional analysis. The first protein extraction was using Tris – HCI buffer and TCA precipitation, second extraction method was the phenolic extraction and ammonium acetate precipitation and the third method was imidazole buffer extraction and TCA precipitation.

Extraction method 1

The extraction method done is derived from studies done by Yang et al (2013) with slightly modification. This extraction procedure had been repeated two times. Firstly, only wild type rice sample with 3 different masses of 0.1g, 0.2g and 0.2g were carried out. Second extraction was for wild type rice sample with 3 different masses of 0.1g, 0.2g and 0.3g but with 3 duplicate each sample. Extraction for irradiated rice sample was not yet been done.

Materials:

30 mL Extraction buffer containing:

20 mM Tris – HCI, pH 7.5

250 mM Sucrose

10 mM EDTA

1 mM PMSF

1 mM DTT

1% Triton X – 100 (v/v)

50% TCA

40 mL Cold acetone containing 13 mM DTT

10 mL Solubilization buffer containing:

4.20g urea

1.52g thiourea

First the rice seeds were peeled and grinded into powder and then the samples were put in labeled tubes. Protein sample weighted 0.1g with 3 replicates each of them labeled A1, A2 and A3. Protein sample weighted 0.2g with 3 replicates each of them labeled B1, B2 and B3. Protein sample weighted 0.3g with 3 replicates each of them labeled C1, C2 and C3. Then 1 ml of Tris - HCI extraction was added into each tube. All samples were then centrifuged at 13, 200 rpm, 4° C, for 10 minutes. After the centrifugation, the supernatant was obtained. Then, precipitation was done with 50% TCA (4:1). Then, the samples were centrifuged again at 13,200 rpm, 4°C, for 10 minutes. After that, the supernatant was poured out and the pellets obtained were kept. The pellet was then washed with 40 mL Cold acetone containing 13 mM

DTT four times. After washing process, the pellets were then air – dried. Finally the pellets were solubilize in 50 uL solubilization buffer each and stored in -20°C freezer.

Extraction method 2

The extraction method done is derived from studies done by Nallamilli et al (2014) with slightly modification.

Materials:

30 mL Extraction buffer containing:

0.5 M Tris – HCI, pH 8.7

0.9 M Sucrose

0.05 M EDTA

1 mM PMSF

0.1 M KCI

2% b- mercaptoethanol (added freshly)

Phenol (prepared using Cold Spring Harbor Protocols)

First the rice seeds were peeled and grinded into powder and then the samples were put in labeled tubes. Protein sample weighted 0.1g with 3 replicates each of them labeled A1, A2 and A3. Protein sample weighted 0.2g with 3 replicates each of them labeled B1, B2 and B3. Protein sample weighted 0.3g with 3 replicates each of them labeled C1, C2 and C3. Then 500 ul of extraction and 500 uL phenol was added into each tube. All samples were then

centrifuged at 13, 200 rpm, 4° C, for 10 minutes. The phenol phase was recovered and the phenol extraction was repeated three times. The final collection of phenol was mixed with five volumes of precipitation buffer. Precipitation was carried out at overnight. The precipitant was recovered by centrifugation at 13,200 rpm, 4° C, for 10 minutes. The pellet was washed three times with cold precipitation buffer and then three times with ice cold 70% ethanol. The pellets were then air – dried. Finally the pellets were solubilize in solubilization buffer each and stored in -20°C freezer.

Extraction method 3

Extraction buffer was based on Nakamura et al (2012) with modification. Functional later action between plastidial starch phosphorylase and starch branching enzymes from rice during the synthesis of branded maltodextrius.

Materials:

Extraction buffer: 50mM imidazole –CI, ph 7.4

8mM mgCI2

12.5% Glycerol

2.5% b-mercaptoethanol

0.1Mm PMSF

Precipitation with 20% TCA/Acetone

Washing with 100% Acetone

First the rice seeds were peeled and grinded into powder and then the samples were put in labeled tubes. Protein sample weighted 0.1g with 3 replicates each of them labeled A1, A2 and A3. Protein sample weighted 0.2g with 3 replicates each of them labeled B1, B2 and B3. Protein sample weighted 0.3g with 3 replicates each of them labeled C1, C2 and C3. 1 mL Extraction buffer was added in each tube. All samples were then centrifuged at 13, 200 rpm,4° C ,for 10 minutes. The supernatant was obtained. Then precipitation was done with 20% TCA/Acetone for 30 minutes. The precipitant was recovered by centrifugation at 13,200 rpm, 4° C , for 10 minutes. Then, the supernatant was poured out, pellet is kept. The pellet was then washed with 100% acetone. The pellets were then air – dried. Finally the pellets were solubilize in solubilization buffer each and stored in -20°C freezer.

3.3 Protein Quantitation using Bradford Method

This protein quantitation is based on Kruger's Bradford method. Duplicate samples containing between 1 and 10 μg in a total volume of 100 μL were pipetted into 1.5 mL polyethylene microfuge tubes. Duplicate volumes of 10, 20, 40, 60, 80 and 100 μL of 100 μ/mL bovine serum albumin standard solution were be pipetted into microfuge tubes for the calibration curve. Then the volume was adjusted to 100 μL with water. 100 μ L of distilled water was pipetted into a tube for the reagent blank. 1 mL of protein reagent was added to each tube and mix gently, but thoroughly. The absorbance of each sample is measured for three times as the