CANDIDATE MUTANTS OF *Solanum Lycopersicum* DEVELOPED USING ETHYL METHANE SULFONATE

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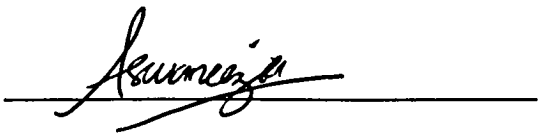

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ABSTRACT

Solanum lycopersicum belongs to the genus of *Solanum* with a small genome size of 950Mb of DNA in 91 scaffold aligned to 12 tomato chromosome. *Solanum lycopersicum* which has been known to be originated from Andean Region and widely used as a research material has contributed towards the vast amount of tomato genetic information such as the discovery of 34,727 protein coding gene and a mass of accumulated information of tomato functional genomics in various databases. However, the functional genomics of plant genes has not yet been fully correlated to its specific function which only 18,320 genes were specified. Despite of having a high economical value with other significant attribution and benefits, its fullness can't be achieved if vital gene function has not been known yet. The present study was performed to correlate the scrutinized targeted plant genes to its function in the basis of *S. lycopersicum* mutagenesis via chemical mutagen ethyl methanesulfonate (EMS). Mutant tomato plants were developed up to its second generation mutant line which its genomic DNA were sequenced with the use of nine primers for the study of functional genomics of the specific loci in the tomato genome. All 99 samples were sequenced and screened for the changes in genotypic aspect through TILLING and reference comparison to its control untreated DNA sequence via bioinformatic software. Based on the analyzed results a total of five genes were detected to be mutated through random mutation induced in seeds by ethyl methanesulfonate treatment. The variations in 12 main classes and 24 sub-classes of tomato phenotypes, Brix sugar index, chlorophyll level and tomato height that were observed and recorded from germination stage up to senescence stage of tomato plants were in line with the polymorphism screened in the sequence of the mutated genes. Sugar index were also relatively low in mutant fruits with a mean value of 3.8 %Brix in comparison to control tomato fruits of 6.2 %Brix, indicating HXK gene might take part in the Carbon catabolite repression (CCR). Mutagenic effect of EMS were notably in the variation of mutant and control plant heights, mutant plant were stunned with the shortest to be recorded with a height of 10 cm of its main stem. Chlorophyll levels were relatively lower in leaves of mutant plants. EXP gene was screened to be mutated and caused the growth of shorter stem and nodes of tomato plants as it regulates the loosening of plant cell wall. Ethyl Methanesulfonate has proved its reliability in plant mutagenesis through the screening of newly emerging technology of TILLING for the study of plant functional genomics and further intensive research can be applied on tomato whole genome. This research of tomato as a plant model could contribute in the agriculture sector that emphasized on *Solanum lycopersicum* and mutagenesis via Ethyl methanesulfonate.

ABSTRAK

CALON-CALON MUTAN Solanum lycopersicum DIBANGUNKAN MENGGUNAKAN ETHYL METHANE SULFONATE

Solanum lycopersicum tergolong dalam genus Solanum dengan saiz kromosom 950Mb asid deoksiribonukleik (DNA) di 91 perancah dijajarkan kepada 12 kromosom tomato. Solanum lycopersicum telah diketahui berasal dari Wilayah Andean dan digunakan sebagai bahan penyelidikan secara meluas, ia menyumbang kepada sejumlah besar maklumat genetik tomato seperti penemuan 34,727 gen pengekodan protein dan sejumlah besar maklumat genomik fungsian tomato terkumpul dalam pangkalan data. Walau bagaimanapun, fungsi genomik gen tumbuhan belum dikaitkan sepenuhnya kepada fungsi khusus mereka, hanya 18,320 gen telah dispesifikasikan. Walaupun ia mempunyai nilai ekonomi yang tinggi dan faedah lain yang penting, kebaikan keseluruhannya tidak boleh dikecapi jika fungsi gen penting belum diketahui lagi. Kajian ini telah dijalankan untuk mengaitkan gen tumbuhan yang dikaji kepada fungsinya secara dasar mutagenesis S. lycopersicum menggunakan Ethyl methanesulfonate (EMS). Tanaman tomato mutan dibiak sehingga generasi kedua di mana DNA genomik mereka telah melalui proses penjujukan DNA dengan menggunakan sembilan primer bagi mengkaji genomik fungsian lokus tertentu dalam genom tomato. Kesemua 99 sampel telah melalui proses penjujukan DNA dan disaring untuk sebarang perubahan dalam aspek genotip melalui kaedah TILLING dan perbandingan kepada jujukan DNA kumpulan kawalan. Keputusan analisis menunjukkan sejumlah lima gen dikesan telah bermutasi melalui kaedah rawatan Ethyl methanesulfonate. Perbezaan pada 12 kelas utama dan 24 sub-kelas fenotip tomato, indeks gula Brix, paras klorofil dan ketinggian tomato yang direkodkan dari peringkat percambahan sehingga peringkat penuaan tanaman tomato adalah selaras dengan polimorfisme yang disaring di jujukan DNA gen yang bermutasi. Indeks gula buah-buahan mutan adalah rendah dengan nilai rata-rata 3.8 %Brix berbanding dengan buah tomato kumpulan kawalan iaitu sebanyak 6.2 %Brix, menunjukkan gen HXK berkemungkinan mengambil bahagian dalam Karbon Repressi Katabolite. Kesan mutagenik EMS adalah ketara terutamanya pada variasi ketinggian tumbuhan mutan dengan kumpulan kawalan, pokok-pokok mutant terbantut dengan ketinggian paling rendah, 10 cm dan paras klorofil juga rendah. Saringan gen EXP menunjukkan ia bermutasi dan menyebabkan pertumbuhan batang dan nod tanaman tomato lebih pendek kerana ia mengawal proses pelonggaran dinding sel tumbuhan. Ethyl Methanesulfonate telah membuktikan kebolehpercayaannya dalam mutagenesis tumbuhan melalui kaedah saringan teknologi TILLING. Kajian genomik fungsian tumbuhan yang lebih intensif boleh dilakukan pada keseluruhan genom tomato. Penyelidikan ini yang menggunakan tomato sebagai model tumbuhan yang boleh menyumbang kepada sector pertanian menekankan penggunaan Solanum lycopersicum dan mutagenesis melalui Ethyl methanesulfonate.

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LIST OF ABBREVIATIONS

AGO	-	Argonaute 6
ATP	-	Adenosine triphosphate
CTAB	-	Cetyltrimethylammonium bromide
CIA	-	Chloroform-isoamyl
CCR	-	carbon catabolite repression
cDNA	-	Complementary DNA
dsDNA	-	Double-stranded DNA
DAF	-	Days after flowering
DNA	-	Deoxyribonucleic acid
EMS	-	Ethyl Methanesulfonate
EXP	-	Expansin
EtBr	-	Ethidium Bromide
GMO	-	Genetically modified organism
HEX	-	Hexokinase
ISSR	-	Simple Sequence Repeat
LD	-	Lethal Dose
LTR	-	Long Terminal Repeat
Mb	-	Megabase
NCBI	-	National center for biotechnology information
ORF	-	Open reading frame
PHYSN	-	Phytoene Synthase 1
PCANH	-	Carbonic Anhydrase
P2D1	-	Photosystem II D1

PHYSN	-	Phytoene Synthase 1
PCANH	-	Carbonic Anhydrase
PCR	-	Polymerase Chain Reaction
RP2	-	RNA Polymerase 2
RNA	-	Ribonucleic Acid
SNPs	-	Single nucleotide polymorphism
TILLING	-	Targeting Induced Local Lesions in Genome
TSS	-	Total Soluble Solid
UV	-	Ultra Violet

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Tomato (*Solanum lycopersicum*) is a fruit-bearing vegetable plant that belongs to the family of Solanaceae which also commonly known as nightshades. Tomato and other related nightshades have been ascertained to be originated from Andean Regionof South America including some areas of Ecuador, Peru and Chile (Sims, 1980). The Solanaceae family comprises more than 3000 species and were said to be the third most important plant for economical proposes. Solanaceae sub-family consists of a variety amount of crop species from potato to medicinal plants which are constantly used as plant model system in agricultural and biological field (Fernandez-pozo*et al.,* 2014). Plant breeding of *S. lycopersicum* is essential in ensuring better and improved characteristic of the plants to meet several necessities and requirements of commercial production. Tomato is chosen as a plant model system in this study as the genome sequence of tomato is relatively small with 950 megabases of DNA in 91 scaffold aligned to the 12 chromosomes found in tomato, this is relatively small compared to the other crops within Solanaceae family (Matsukura*et al.,* 2008), making tomato an ideal reference species for sequencing the genome of Solanaceae family (Emmanuel & Levy, 2002). SOL genomic network had managed to discover 34,727 protein coding gene (The tomato genome Consortium, 2012). Most of the plants from Solanaceae family exhibit genomic stability (Doganlar*et al.,* 2002). However, the gene function of tomato has not been fully studied with only 18,320 known gene correlated to its specific function. The development of mutant tomatoes by means of mutagenesis could facilitate the study of plant gene function (Reddaiah*et al.,* 2014) through the approach of newly emerging technology of TILLING (Gilchrist &Haughn, 2005; Stemple, 2004) for the search of mutation on the specific studied genes.

Tomato is seen to contain high amount of vitamin which is mainly ascorbic acid, vitamin A, potassium, micro and macro nutrients.The high demand of tomato due to it's commercialize value and contribution to medical health benefits such as the presence of relatively high content of antioxidant particularly lycopene has necessitated the need to develop novel, high-yielding varieties of tomato plants (George *et al.,* 2004) and for the purpose of functional genomics (Emmanuel & Levy, 2002). Other than the relatively small genome of tomato and its thousands of discovered genes, *S.lycopersicon* is also specifically chosen for its fairly short life cycle which makes it a convenient plant to study up to their second generation. Tomato tree does not consume a lot of space and suitable to be grown with a constant parameter in greenhouse or transgenic facility. Novel varieties of tomato crop can be breed through four fundamental approaches namely conventional plant breeding, protoplast fusion, genetic engineering and mutation breeding.

Breeding programs of tomatoes such as conventional plant breeding, genetic engineering and protoplast fusion are the approaches to breed better or improved varieties of plants. These various approaches had led to tomato crops with commercially targeted traits such as texture and taste enhancement, enhanced growth, resistance to certain diseases and other useful quality traits. Despite of these approaches in the development of new varieties of crops plants, there are some issues that were consider as drawback specifically the negative prospect in regards to ethical issue.

Regulatory issues pertaining to genetically modified organism has been a main reason for the addition of mutagenesis as an alternative approach in functional genomics of S. *lycopersicum.* The approach of developing new verities of crops in a more rapid and easy way by means of mutation breeding was firstly discovered in 1930s (Schouten & Jacobsen, 2007). Techniques of mutagenesis breeding were then improved after World War II. The research approach adopted in this study focuses on chemical mutagenesis breeding via exposure of Ethyl Methanesulfonate (EMS) which is an alternative approach to genetic engineering. Chemical mutagen such as Ethyl Methanesulfonate (EMS) which acts on guanine, is capable to produce mutation in diploid DNA with the range of two to 10 mutation per megabase (Mb) (Till *et al.,* 2007). Although mutagenesis breeding is more randomized compared togenetic modification of plants (Lai *et al.,* 2004), mutagenesis is a relatively

conventional approach as it needs a fewer amount of plantlets, time and cost reduction in yielding high number of tomato plants (Kim *et al.,* 2006; Clark, 1963; Li *et al.,* 2016). The development of mutant population with significant change in phenotypes and physiological responses is a robust tool in the study of biological function of specific genes in plants. The use of chemical mutagen, EMS aided with new technology of Targeting Induced Local Lesions in Genome (TILLING) are the approaches used for the study of functional genomics in tomato plants. TILLING is the best approach used for the detection of mutation developed through EMS in terms of the reduced amount of reagent consumed, duration in producing a mutant population, high density of mutation to the work load in screening for target genes (Serrat*et al.,* 2014)which firstly tested on *Arabidopsis thaliana* (McCallum *et al.,* 2000) and mutation on *Drosophila melanogaster* (Bentley *et al.,* 2000). The functional genomics of the specific genes will be analyzed through the development of mutant plants via EMS and TILLING approach (Kim *et al.,* 2006) through the analysis in both of the plant's genomic and phenotypic aspect. The basis of TILLING involves the development of mutant population and ends with the screening and mutation discovery on the specific studied gene (Comai&Henikoff, 2006). TILLING has been an established technology in mutagenesis breeding for genetic studies linking genes with their associated functions.

1.2 Objectives

The research objectives of this study are:

i. To develop a mutant population of *S.lycopersicum* via chemical mutagenesis.
ii. To screen the population for mutant on the basis of phenotype.
iii. To evaluate the effect of chemical mutagenesis on specific genes.

1.3 Significance of study

The discovery of thousands coding protein in tomato plant genome is one of the greatest achievements in plant genetics. Various research papers were published in regards to the number and types of new genes found in tomato, recently more than eighteen thousand genes were knownpublicly. Although this had lent a tremendous improvement in plant study, there are still a few genes have not yet been link to their specific function. This research approaches focus on the study of functional genomics in tomato as a model plant via TILLING technology. Mutation occurs in the targeted gene will aid in determining the function of studied gene. The study of gene function could bridge the gap between the function of the gene or gene regulation within the plant genome. The function of mutagenesis is proven for the study of reverse genetics through the screening of mutation along the target genes by means of DNA sequencing. The knowledge through this research could be useful for the study of this model plant and the mutagenic effect of chemical mutagen particularly EMS. This will benefit the agriculture sector that emphasize on the study of this model plant and gene regulation.

CHAPTER 2

LITERATURE REVIEW

2.1 *Solanum lycopersicum*

Tomato (*Solanum lycopersicum*) originated in South America and the commercialization of this particular plant continues to dominate other countries due to its usefulness as a vegetable crop. The anatomic features of tomato consist of its unique compound leaves, differential texture of its fruit and sympodial shoot branching. Furthermore, the tremendous advantages of tomato also include its short life cycle, self-pollination and non-complex diploid genetics.

The word Tomato was originated from a Spanish word tomate where its species originally grown in the South American Andes. The large amount of tomato consumed made it classified as the second highly intake vegetable (Szczechura*et al.*, 2011). Tomatois one of the crops that has been harvested for the purpose of commercialization and contribute as a model plant for increasing the depth in knowledge for the aspect of growth, maturation and metabolism in the biology of fruits (Giovannoni, 2004; Carrari*et al.*, 2006) and considered as a good plant model system for Solanaceous family due to its considerably small genome size (Reddaiah*et al.*, 2014). The whole tomato genome has been successfully sequenced by The International Solanaceae Genomics Project (SOL) (Muller, 2005a; Muller 2005b). It's genome consisted of more than 75% of heterochromatin (Fernandez-pozo*et al.*, 2014) and 730 Mb of the tomato genome are pericentromeric heterochromatin with 220 Mb of the tomato chromosome made up of distal euchromatic segments (Szczechura*et al.*, 2011). Tomato genome was initially thought to be a huge challenge for full sequencing as its genome is complicated and larger in size

compared to animal model, it was then discovered that tomato genome in the range of 950MB is considered manageable.

The Tomato Genome Sequencing Project has been one of the most important projects in the history of sequencing, this project employed both Sanger's and next-generation sequencing (NGS) technologies for the assembly of plant genome as well as a draft of *Solanum pimpinellifolium*which was released in 2012 (TGC, 2012). The sequence data generated by 150 tomato Genome Consortium has further uncover the natural alleles exist in different genotypes of tomato (Mueller *et al.,* 2009). However, the functional potential of the major fraction of this newly generated resource is still undefined.

Tomato has been used for the study of functional genomics in plants. *Arabidopsis thaliana* was initially the main model plant for this purpose due to its significant characteristics advantages. With the identified similar leverage of small genome size and short life cycle of tomato in comparison to *Arabidopsis thaliana,* makes tomato an ideal plant for gene study (Watanabe *et al.,* 2007).

Research on tomato were previously conducted with various tomato varieties including Arka Vikas for the purpose of investigating the effect of mutagenic treatment on agronomic parameters such as seedling heights and plant height (Laskar*et al.,* 2016), Patharkutchi was used to study the frequency and spectrum of macro-mutations of EMS and its combined treatment (Dutta *et al.,* 2017), Red Setter for the reverse genetic study in creating new traits of specified variety (Minoia*et al.,* 2010) and *Lycopersiconesculentum* Mill. was chosen as the model plant for the study of effects of mutagen concentration (Aliyu &Adamu, 2007). Studies of these tomato varieties focuses on the use of ethyl methanesulfonate as the chemical mutagen however, tomato variety of Yates F1 Hybrid (Grosse Lisse) from Australia has never been studied before. Thus, Yates F1 Hybrid (Grosse Lisse) was chosen in this research to study the mutants developed from this specified variety via EMS mutagenic treatment.

2.2 Breeding Program of Tomato

Conventional plant breeding is one of the approaches to breed better or improved varieties of plants by means of selecting desirable traits and cross breeding between varieties. Newly improved next generation population could be made possible through this method which carries the best traits from the parental plants. Commercially targeted traits were texture and taste enhancement, resistance to certain diseases and additional nutrient values. Conventional plant breeding method starts with specifically selected traits possessed crops via pure-line selection and hybridization between crops for the breeding of future generation. Conventional plant breeding relies on sexual recombination (Manshardt, 2004). Although this step has greatly contributed in the development of new variety of crop plants with inherited gene of interest but the good traits of the parental are only passed down through multiple back-crossing between progeny and recurrent parent which is considered as a time consuming plant development method (Hoisington *et al.,* 1999)

Protoplast fusion in plants on the other hand uses the application of fusing two or more protoplast to transfer useful genes. This approach of genetic recombination is able to transfer diseases resistance, enhanced growth, heat and cold resistance and other useful quality traits for forming good varieties of plants. The process of making new plant hybrids through protoplast fusion requires the removal of cell wall and cell fusion via electrofusion as the prime step (Grosser & Gmitter, 2011). The successful fused cells will have to be grown into calluses, plantlets and finally develop into a fully grown crop (Salamiah *et al.,* 2000). This important fundamental approach uses genetic modification method and requires a few successful steps to efficiently transfer genes between protoplast cells, making it a higher chance of failure in transferring the targeted gene, moreover longer period is required for the regeneration of whole plant (Marcone *et al.,* 2010; Guan *et al.,* 2010)

Genetic engineering applies the concept of preserving the genetic material of parent by only inserting minute genetic information for expressing the chosen characteristics (Richard, 2004).Restriction enzyme aids in the separation of purifiedDNA into fragments in order to isolate the candidate gene (Nicholl & Desmond, 2008; Alberts *et al.,* 2002) or by means of polymerase chain reaction

(PCR) to amplify the gene segment (Kaufman & Nixon, 1996) and extracted through gel electrophoresis. The isolated gene is then ligated into a plasmid and inserted into a and inserted into a bacterium. *Agrobacterium tumefaciens*, a plant-pathogenic bacterium is the widely used bacteria for the insertion of desired traits in genetic breeding. It is well known for its ability to transfer a part of its genetic material into many plant species (Gelvin, 2003). Despite of the high reliability of the commercialization of genetically modified tomato has been completely stopped in the 20th century for some countries due to complications that occurred and expensive patent system related to transgenic plants and consumers concern in various aspects especially in regards to ethical issue (Bai &Lindhout, 2007). Moreover, the insertion of single transgene does not able to provide full benefits for important traits with commercial value due to the complexity of mostly commercially important traits (Strauss, 2003).

2.3 EMS as Chemical Mutagen

Several approaches has been developed and introduced with the purpose of inducing genetic variation among crops for the past century (Smartt & Simmonds, 1995). Initially the development of mutant organisms was conducted by H.J. Muller through the exposure of X-ray on *Drosophila,* increasing its degree of mutation to 15000% (Muller, 1927). Mutagenesis has tremendously reducedthe time needed for natural mutation to occur for it requires no any external source that could induce any form of genetic mutation. The development of mutant plants can facilitate the study of the unknown gene functions (Reddaiah*et al.,* 2014). Various ways has been successfully develop to induce mutation within plant's genome namely exposure to Ultra Violet (UV) rays, Ethidium Bromide (EtBr), fast neutron, transposons, T-DNA and Ethyl Methanesulfonate (EMS).

Chemical mutagent such as EMS was applied in this research asit is a reliable mutagenesis inducer and provides high density of mutant population (Henikoff&Comai, 2006). It is proven that EMS caused point mutation with a few

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