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# Ethyl Methanesulphonate (EMS)-Mediated Mutagenesis Induces Genetic and Morphological Variations in Eggplant (*Solanum melongena* L.)

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**Abstract:** Eggplant (*Solanum melongena* L.) is a vegetable that holds high economic and nutritional value and is renowned for its distinct shape, color and flavor. There has been a considerable focus on enhancing the genetic makeup of eggplant, with specific attention given to breeding for better agronomic characteristics. However, the crop suffers from a narrow genetic base. As part of the efforts to broaden the gene pool of eggplant, a chemical mutagenesis approach has been employed, aimed to generate eggplant genotypes with distinctive characteristics. Altogether 180 seeds of eggplant cultivar, Surya was treated with EMS at 0.7% *v*/*v* concentration. In the development of M2 generation, members of 16 M2 families were inspected for phenotypic variation. Notable variations were observed in traits such as plant height, leaf, flower and fruit morphologies. Furthermore, a subset of the mutants was selected to screen for any DNA alterations in a few targeted genes belonging to the *Flowering Locus T*/*Terminal Flower 1* (*FT*/*TFL1*) gene family, via amplicon sequencing performed using Pacbio RSII. A mutant sample was discovered to carry a heterozygous mutation at the upstream region of the coding sequence of one of those particular genes. Taken together, the M2 families developed here represent valuable genetic resources that can be explored for gene functional analysis and future breeding programs of eggplant.

**Keywords:** eggplant; *Solanum melongena*; mutagenesis; Pacbio RSII amplicon sequencing



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## 1. Introduction

Eggplant (*Solanum melongena* L; 2n = 24) is a crop species that thrives in tropical and subtropical regions. It is known by various regional names, such as brinjal, aubergine, melanzana, garden egg and patlican [1]. It is a crop of economic and nutritional importance. The worldwide production of eggplant amounted to approximately 58.6 million tons in 2021 [2]. Eggplant offers a wide array of health benefits due to its rich nutrient composition, which includes various beneficial components, such as phenolic compounds, including anthocyanin and chlorogenic acid (CGS), proteins, dietary fiber, minerals and vitamins [3]. Moreover, its polyunsaturated fatty acids exhibit a decholestrolating effect, making it useful for treating asthma and liver problems [4]. Its high fiber and low soluble carbohydrate content make it a recommended dietary choice for managing type 2 diabetes and hypertension, as endorsed by organizations such as the National Diabetes Education Program of the NIH, the Mayo Clinic and the American Diabetes Association [5]. Due to its fruit phenolic component, eggplant is also ranked among the top 10 vegetables with oxygen radical absorbance capacity [6].

In recent years, there has been an acceleration of biotechnological interventions in eggplant breeding, particularly in genetics and genomics, aimed at developing improved varieties of the crop [7]. This trend is in line with the growing global interest in eggplant. However, one of the major obstacles to achieving these goals is the narrow genetic diversity of cultivated eggplant despite its wide morphological variation. This variation is believed to have arisen from domestication in Southeast Asia centuries ago [8]. A recent genome-wide

analysis of variations in eight domesticated eggplants and one wild species of *Solanum incanum* revealed that *S. melongena* accessions carried only 0.8 to 1.3 million variants, while more than 9 million were found in *S. incanum*. This finding confirms the limited gene pool of eggplant cultivars and highlights the potential benefits of increasing the genetic diversity of the crop through breeding programs [9].

Mutagenesis, a process that involves artificial modifications to DNA, is a potent technique for accelerating the genetic improvement of eggplant cultivars compared to natural mutations. The occurrence of spontaneous mutations in higher plants is rare, with a frequency ranging from $10^{-5}$ to $10^{-8}$. As a result, mutagenesis has emerged as a highly effective method for creating novel genotypes with enhanced and desirable quality traits. It has also proven valuable for studying gene functions, particularly with the support of accessible genomic resources [10]. Mutagenesis can be achieved by three primary agents: (1) physical agents, such as gamma rays, X-rays and UV light, as well as particle radiation, such as fast and thermal neutrons and beta and alpha particles; (2) chemical mutagens, such as ethyl methanesulfonate (EMS), N-nitro-N-methylurea (NMU), hydrogen fluoride (HF) and sodium azide (SA); and (3) biological agents, such as T-DNA, transposons and retrotransposons [11].

Chemical mutagenesis is an economical, convenient and simple way to induce allelic diversity in plant and animal genomes. There are several chemical mutagens that produce different types of DNA lesions, including alkylating agents and azides [12]. Among these mutagens, EMS (C3H8O3S) is highly preferred and effective in plants due to its high mutagenicity and ease of handling compared to other chemicals, such as nitroso compounds. EMS predominantly induces single base point mutations [13]. As an alkylating agent, EMS transfers an alkyl group to guanine bases, producing 6-ethylguanine, which pairs with thymine instead of cytosine during DNA replication, resulting in G/C to A/T transitions [12]. Previous studies of EMS-induced variations have reported G/C to A/T transitions to occur in more than 99% of cases in *Arabidopsis* [14], 79.8% in maize [15] and 70% in rice [16]. The degree of G/C to A/T transitions may vary according to different plant species due to their genetic compositions and genomic features [17]. EMS also induces G/C to C/G or G/C to T/A transversions or A/T to G/C transitions to a lesser extent, as well as base insertions and deletions [18,19].

The development of eggplant mutant libraries has been rather limited compared to other members of *Solanaceae*, such as tomato and pepper. For example, there are various mutant libraries available for tomato in different backgrounds, such as Micro-Tom, M82, Red Setter and Tpaadasu [20]. Although the focus on improving eggplant through EMS mutagenesis has been relatively limited, there have been significant instances in which mutagenesis has been utilized to generate innovative germplasms. This approach has led to the production of promising materials in eggplant, exhibiting diverse agronomic traits such as plant height, flower morphologies, fruit shapes, sizes, anthocyanin content and insect resistance [20,21]. These materials serve as promising resources for breeding new eggplant varieties and studying gene function [20].

Considering the facts above, the present study aimed at investigating the morphological effects of EMS induction on an eggplant cultivar, Surya. The study was also further extended to evaluate the utilization of targeted amplicon sequencing based on the Pacbio RSII platform to detect the presence of any point mutations at a few selected genetic loci.

## 2. Materials and Methods

### *2.1. Seeds*

Seeds of the Surya eggplant cultivar were procured from the World Vegetable Center (WVC) in Taiwan after receiving import permission from the Sabah Agricultural Department of Malaysia. The identification number of the sample is VI045276 per the records maintained in the WVC. The Surya eggplant cultivar is a commercial and bacterial wilt-resistant variety that provides rootstocks for the analysis of bacterial wilt management in eggplant [22]. It has been characterized to have medium-sized, oval and glossy fruits

(http://www.celkau.in/crops/vegetables/brinjal/brinjal.aspx#3, accessed on 14 July 2023). Hence, it is a suitable candidate for crop improvement programs, especially with high-yielding quality and a bacterial wilt-resistance genotype [23].

Since only a limited number of WVC seeds were available for mutant development, a representative eggplant kill curve analysis was performed using F1 hybrid seeds of a local commercial eggplant cultivar.

### *2.2. Construction of Plant 'Kill Curve' Analysis*

The 'kill curve' analysis was carried out following the documented procedures with slight modifications [24,25]. A total of 100 seeds, divided into six batches, were used for the experiment, with each batch corresponding to different EMS concentrations (0%, 0.2%, 0.4%, 0.6%, 0.8% and 1.0% (*v*/*v*)). Prior to the experiment, the seed samples were soaked in sterile water for 8 h. The seeds were then immersed in their respective EMS concentrations and incubated with gentle shaking for 10 h in the dark at room temperature. Then, the seeds were washed six times with distilled water over a period of 1 h and 30 min. Subsequently, they were placed on Whatman paper in Petri dishes and incubated in a growth chamber at 25 °C with an 8-h photoperiod. The germination was observed for 27 days with the results recorded. The ideal concentration was determined based upon the assessment of the germination of the seeds [24].

### *2.3. Development of M1 and M2 Generation of Eggplant Libraries*

EMS treatment was applied to approximately 180 seeds from the Surya eggplant cultivar using the protocol described for the "kill curve" analysis. A concentration of 0.7% EMS was selected to induce mutagenesis in the seeds. Next, the treated seeds were sown and grown in nursery trays. The seedlings were transplanted into individual polybags in the field with spacing of 50 cm × 50 cm between polybags. Altogether, 40 M1 plants were self-pollinated, and the seeds of mature fruits were harvested and saved for future use. In the subsequent generation, about 95 M2 plants from 16 M2 families were planted, with approximately seven members from each family except for one M2 family (Identity: 46), which was represented by one individual. Additionally, the frequency of the mutant plants relative to the total number of individuals per M2 family was calculated.

The M1 generation was initiated in August 2015, while the subsequent development of the M2 generation was conducted in September 2018. The plants from both generations were cultivated in an experimental field plot located in the Biotechnology Research Institute, Universiti Malaysia Sabah (21°10′2″ N; 110°16′34″ E).

### *2.4. Detection of Genotypic Variation among the M2 Mutant Population*

Of the 95 M2 plants, 16 were chosen to undergo screening for potential changes in their genetic makeup. Within this group, six genes belonging to the *Flowering Locus T* (*FT*)/*Terminal Flower 1* (*TFL1*) family [26] were selected for further analysis. These genes included *TFL1*/*Centroradialis* (*CEN*)-like genes, namely *SmTFL1*, *SmCEN*-1, *SmCEN*-2 and *SmCEN*-4, as well as *SmMFT*-1 and *SmMFT*-2, which are members of the *Mother of FT and TFL1* (*MFT*) gene cluster of the *FT*/*TFL1* gene family.

Leaf samples from both the mutants and controls were used to extract genomic DNA for amplicon generation. This goal was accomplished using a CTAB-based method modified from Webb and Knapp [27]. The mutant samples, as well as three control plants, underwent amplicon generation of *SmTFL1*, *SmCEN*-1, *SmCEN*-2, *SmCEN*-4, *SmMFT*-1 and *SmMFT*-2. The multiplexed amplicon sequencing utilized an asymmetric barcode system, which involved different combinations of barcodes attached to the forward and reverse primers. Each plant's homologs were assigned a unique barcode combination. The barcodes were introduced to the amplicons via a two-step PCR protocol. The first step, PCR1, involves PCR primers with universal and gene-specific sequences (GSPs) to amplify the genomic DNA template. The GSPs were designed from the upstream and downstream regions of the coding sequences, as derived from the respective parental sequences (genome assembly).

As such, some of the amplicons, notably the *SmMFT*-2 gene, had a few upstream nucleotides sequenced for which mutation detection analysis was included, although sequences from the start to stop codons were primarily targeted for all sequences. In the second step, PCR2, barcodes were introduced to the amplicons using primers with universal and barcode sequences. This PCR was performed on the amplicon template generated in PCR1, allowing for the creation of uniquely identified amplicons for each sample. The Supplementary Material File contains the list of primers utilized, which can be found in Tables S1–S3.

The Kapa HiFi HotStart ReadyMix PCR kit was used to conduct two-step PCR. Each reaction (25 µL) consisted of 1x reaction buffer, 0.3 µM of both the forward and reverse primers and approximately 50–100 ng of genomic DNA. The cycle parameters were approximately 1 min per 1-kb gene at 95 °C, followed by 30 cycles of 20 s at 98 °C and 15 s at 60 °C and a final extension of approximately 1 min per 1-kb gene at 72 °C. In the second round of PCR, barcodes were attached to the amplicons using identical conditions except that 10–20 ng of DNA template (amplicons from the first round of PCR) was used.

The amplicons were combined in equal amounts and purified before being used to construct an SMRTbell library. The starting sample pool was 1.26 µg, and standard procedures were followed for SMRTbell adapter ligation. Sequencing was conducted using P6v2C4 chemistry with a six-hour movie time.

### *2.5. Pacbio Sequence Data Processing*

The sequencing data received in bax.h5 files were converted into bam files using the bax2bam (version 0.0.9) program and were demultiplexed using the pblima (version 1.11.0) program. The phased amplicon sequences were obtained with the pblaa (version 2.4.2) program, which provides subread coverage for every allele. The total number of unique ZMWs in the pool of subreads representing every allele was determined to generate amplicon coverages, as every subread's identity includes the identity of the ZMW from which it was produced. The gene homologs were aligned using the ClustalW program, and nucleotide variations were screened by comparing them to the control sequences, via Mega software, version 10.2.6 [28]. The gene prediction tool Fgenesh (http://www.softberry.com/, accessed on 3 October 2022) was used to establish the position of any detected mutations in the gene with the gene-finding parameter set to tomato, the closest relative of eggplant.

### *2.6. Morphological Screening of Unique Variations Emerging from the M2 Mutant Library*

To evaluate the impact of EMS mutagenesis-induced lesions on phenotypic changes among the M2 plants, several characteristics were examined to determine their plant growth and appearance. This process included the assessment of the plant height and leaf structures, as well as examining traits related to flower and fruit morphologies. The plant height was measured from the base to the terminal of the main stem. The leaf area was measured from the fifth leaf of each plant. The changes in plant morphologies were recorded and imaged using a digital camera.

## 3. Results

### *3.1. Plant 'Kill Curve' Analysis*

The "kill curve" analysis was developed by assessing the germination percentage of representative eggplant seeds (F1 hybrid), as shown in Table 1. The experiment included an untreated seed batch, which exhibited a significant decline in endogenous seed viability with pre-soaking. The range of germination for concentrations up to 0.4% EMS, including the control batch, was narrow. However, a substantial decrease in germination, with a 66% kill rate compared to a 49% kill rate at 0.4% EMS, was observed at 0.6% EMS, indicating that EMS is highly effective in inducing relatively high mutation density at this concentration. Notably, concentrations between 0.6% and 0.8% EMS displayed minimal variation. The highest reduction in germination was observed at 1.0% EMS and was therefore not considered.

**Table 1.** Kill Curve Analysis.

| EMS Concentration (% *v*/*v*) | Germination Percentage (%) |
|---|---|
| 0 | 58 |
| 0.2 | 53 |
| 0.4 | 51 |
| 0.6 | 34 |
| 0.8 | 30 |
| 1.0 | 6 |

Based on these findings, the concentration range between 0.6% and 0.8% EMS was selected as the ideal range for inducing a relatively high mutation density without excessively compromising plant viability. Moreover, the desired survival rate based on "kill curve" analysis was reported to be within the range of 30–80% [12]. The survival rate in comparison to the control at this concentration range is between 52% and 58%. Based on the results of the "kill curve" analysis, it was decided to use 0.7% EMS to induce mutations in the Surya cultivar since it falls within the range of 0.6% to 0.8% EMS.

This 'kill curve' analysis provided a general representation of the EMS concentrations and the survival rate for the *S. melongena* L. germplasm, as established with the mutagenesis protocol applied in this study. The use of a pre-existing set of ideal conditions for different varieties of a species has been documented in some crops [29]. However, it is important to note that the applicability of these conditions can be influenced by potential variations among different varieties [30]. Therefore, in this study, the effects posed by the chosen concentration of EMS on the experimental material (cultivar: Surya) were pre-evaluated by assessing the germination rates before proceeding with field cultivation. Overall, the 180 seeds treated with EMS had a germination rate of 28.9%, while a batch of 40 untreated control seeds had a germination rate of 72.5%. In addition to the robust reduction in germination observed in the EMS-treated seeds, subsequent screening of morphological variations also enabled the assessment of these EMS-mediated effects in generating morphological variations, which could be used in developing variabilities in the germplasms.

### *3.2. Genotypic Screening of M2 Mutants*

We attempted to screen for mutations induced by EMS mutagenesis by targeting six selected genes belonging to the *FT*/*TFL1* family [26]. The amplicons of the genes were derived from the control and mutant samples and were subjected to Pacio RSII-targeted amplicon sequencing. The output is shown in Table 2. The mutations were then identified by comparing the mutant sequences with their respective controls. The full-length sequences of the genes from the start to stop codons (inclusive of exons and introns) derived from the control samples of the Surya cultivar have been deposited in NCBI Genbank under the following accession numbers: *SmTFL1* (OQ025058) (2620 bp), *SmCEN*-1 (OQ025060) (1015 bp), *SmCEN*-2 (OQ025061) (2596 bp), *SmCEN*-4 (OQ025057) (1488 bp), *SmMFT*-1 (OQ025063) (2134 bp) and *SmMFT*-2 (OQ025065) (981 bp) [26].

**Table 2.** Sequenced *FT*/*TFL1* Gene Sequences.

| ***FT*/*TFL1* Amplicons** | **Output (Mutants)** |
|---|---|
| *SmCEN*-1 | 10 |
| *SmCEN*-2 | 12 |
| *SmCEN*-4 | 13 |
| *SmTFL1* | 6 |
| *SmMFT*-1 | 11 |
| *SmMFT*-2 | 14 |

After analyzing the data, we identified a heterozygous mutation in the 5′ untranslated sequence region of the *SmMFT*-2 gene from a mutant sample. The structure of the gene

was determined by a gene prediction tool, Fgenesh (Supplementary Material, Figure S1). This mutation was located 36 bases upstream from the start codon and was found to be a T to C substitution. The mutation had a high accuracy of 1. The predicted accuracy of the consensus sequence, as determined by the pbLAA software, was calculated by multiplying together the quality values (QVS) generated. The details are outlined in Table 3. The region of mutation is depicted in Figure 1. The full-length alignment is illustrated in the Supplementary Material (Figure S2).

**Table 3.** Details of Mutations Screened in *SmMFT*-2 Gene Amplicon.

| *SmMFT*-2 (Mutant Sample) | Amino Acid | Predicted Region | Amplicon Coverage | Predicted Accuracy (pbLAA) |
|---|---|---|---|---|
| Allele_31-3_1 | T | 5′UTR | 77 | 1 |
| Allele_31-3_2 | T > C | 5′UTR | 72 | 1 |

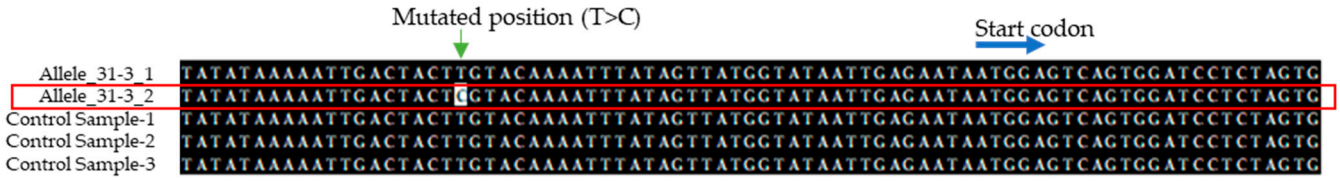


**Figure 1.** Mutation identified in the upstream region of *SmMFT*-2 gene of mutant sample 31-3. The mutated base is displayed in the white background, and the heterozygous allele carrying the mutation is indicated by the red box. The black background shows regions with identical bases. The blue arrow indicates the region of the start codon (ATG). The green arrow shows the location of the mutation, which occurs 36 base pairs upstream from the start codon.

This approach involving mutation detection among the amplicon sequences of mutant samples follows a reverse genetic strategy of TILLING (targeting-induced local lesions in genomes). Consequently, further validation is required to determine the effect of the mutation in the predicted 5′UTR on the subsequent generation, especially on the traits influenced by *MFT* genes. These genes were reported to influence flowering regulation and seed traits [31]. Since the effects of a heterozygous mutation may not lead to observable traits, it is essential to screen sufficient samples within the M3 family to recover the mutation in homozygous state. This process could allow for a comprehensive analysis of its effects [32,33].

### *3.3. Morphological Screening of Unique Variations in the M2 Population*

To evaluate the effects of EMS-induced lesions among the mutant population, various phenotypic characteristics were assessed, including plant height, leaf structures and fruit and flower morphologies, as outlined in Table 4.

#### 3.3.1. Plant Height

Plant height specifically was subdivided into several sections, as shown in Table 4. The evaluation revealed a widespread impact of EMS mutagenesis on this trait, with the majority of the mutant family showing changes in height. Notably, a dwarf mutant (sample 9-1) was identified, with a height of less than 10 cm. As depicted in Figure 2, this mutant also exhibited smaller leaves and fruits compared to the control plants, which ranged from 38.1 to 48.9 cm in height. On the other hand, the tallest mutant of the Surya cultivar, designated as mutant 33-5, stood at a height of 75.9 cm and had the largest leaf area, measuring 416.4 $cm^2$. While some mutants with modified height showed an expected impact on leaf size, other mutants with altered height appeared to be independent of the leaf characteristics and vice versa.

**Table 4.** Morphological Variations Observed During M2 Mutant Screening.

| First Category | Second Category | No. of Mutants | Mutant Family |
|---|---|---|---|
| Plant height (cm) | 1 Dwarf (<10 cm) | 1 | 1 |
| | 2 Very short (10–19 cm) | 2 | 2 |
| | 3 Short (20–38 cm) | 48 | 15 |
| | 4 Normal (38.1–48.9) (N = 7) | 25 | 14 |
| | 4 Tall (50–60 cm) | 11 | 10 |
| | 5 Medium tall (61–70 cm) | 1 | 1 |
| | 6 Very tall (>70 cm) | 1 | 1 |
| Size of leaves ($cm^2$) | 1 Too small (<50 $cm^2$) | 4 | 4 |
| | 2 Medium Small (50–100 $cm^2$) | 7 | 6 |
| | 3 Small (101–204 $cm^2$) | 34 | 14 |
| | 4 Normal (205–300 $cm^2$) (N = 7) | 24 | 14 |
| | 5 Big (301–400 $cm^2$) | 3 | 3 |
| | 6 Very Big (>400 $cm^2$) | 2 | 2 |
| Leaf shape | Curled leaves | 1 | 1 |
| Leaf structure | Thick and shiny, reduced in size | 1 | 1 |
| Leaf color mutation | Light green | 2 | 1 |
| Color of fruits | Bright purple | 1 | 1 |
| | Even coloration | 1 | 1 |
| Shape of fruits | Elongated and/or curved | 3 | 3 |
| Flower morphology | Variation in petal formation | 2 | 2 |
| Flower color | Bright purple | 1 | 1 |

Note: The category known as "normal" pertains to measurements that fall within the range exhibited by the controls. The notation "N = 7" signifies the utilization of seven control samples for morphological evaluations.

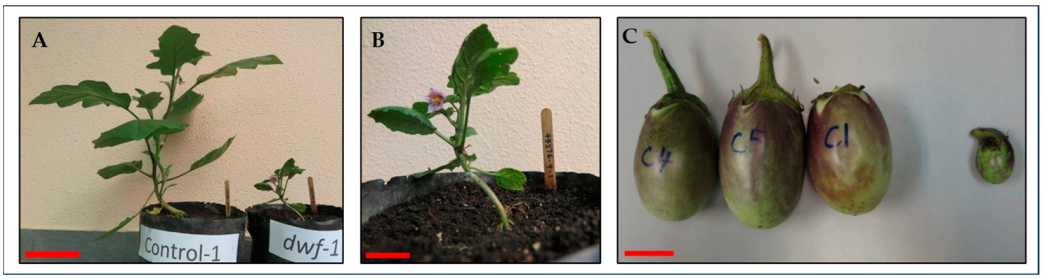


**Figure 2.** The dwarf mutant sample 9-1. Images (**A**,**B**) represent the front and enlarged views of the mutant plant, respectively, while image (**C**) shows the mutant fruit. Per the images (**A**,**C**), the mutant samples are displayed on the right, while their control images are displayed on the left. The scale bars are 10 cm for image (**A**) and 3 cm for images (**B**,**C**).

### 3.3.2. Leaf Structure

Among the mutants of the cultivar, some showed variations in leaf color and morphologies, as presented in Figures 3 and 4. Mutant plant 36-5 displayed altered leaf coloration, showing lighter shades of green (as illustrated in Figure 3). Plant 39-6 exhibited curled leaves, specifically exhibiting downward curling. Furthermore, Figure 4 shows an interesting display of a mutant (31-6) that emerged with 'glossy' leaves.

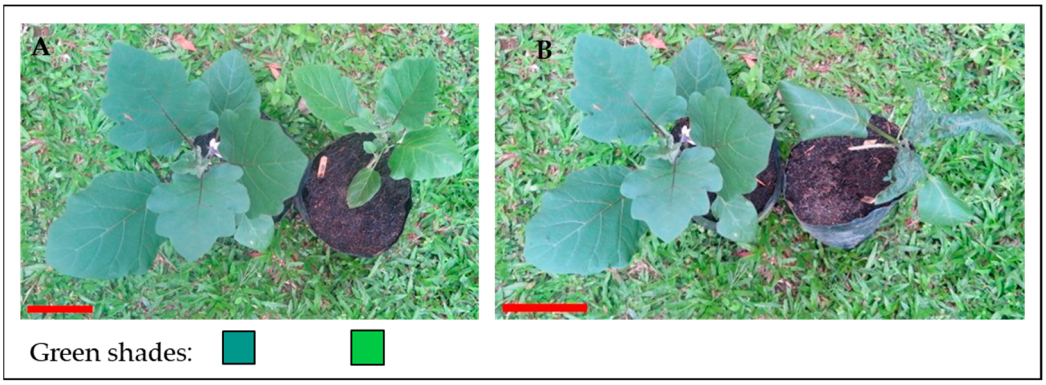


**Figure 3.** Variations observed in the leaf structures. Images (**A**,**B**) denote mutant samples 36-5 and 39-6 with leaf color mutation and curled leaf structures, respectively. Images display control samples on the left and mutant samples on the right. Scale bar is 10 cm.

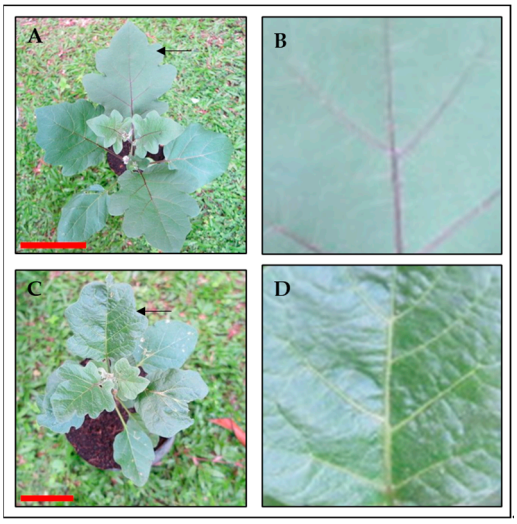


**Figure 4.** Development of 'glossy' leaf trait of mutant sample 31-6. Images (**A**,**C**) denote control and mutant plants, respectively. Images (**B**,**D**) display the enlarged views of the leaf samples of control and mutant plants, respectively. Emergence of 'glossy' leaf trait is visible on images (**C**,**D**). The arrows in images (**A**,**C**) point to the leaf samples that were enlarged, corresponding to images (**B**,**D**), respectively. Scale bars are 6 cm for image (**A**) and 3 cm for image (**C**).

#### 3.3.3. Fruit Mutants

Interestingly, a number of mutants displayed alterations in fruit shape, as demonstrated in Figure 5, in which deviations from the typical oval shape of the fruit were observed, ranging from oblong to variations in the indented curvature. Moreover, the study also identified fruit mutants with modified pigmentation of a deep purple hue, in stark contrast to the fruit peel pigmentation of the control plants.

#### 3.3.4. Flower Structures

In addition, the study also uncovered variations in the morphology and color of flowers, as depicted in Figure 6. Mutant sample 31-3 showed a striking difference in the flower color, with enhanced purple pigmentation. Meanwhile, mutant plant 31-1 showed changes in flower structure, displaying reduced numbers of petals.


**Figure 5.** Variations observed in the fruit characteristics of the mutant samples. Images (**A**–**D**), which show mutants 46, 39-3, 33-7 and 18-2, respectively, depict fruit color and/or shape mutations. Control samples are displayed on the, left and mutant samples are displayed on the right in all images. Scale bar is 6 cm.

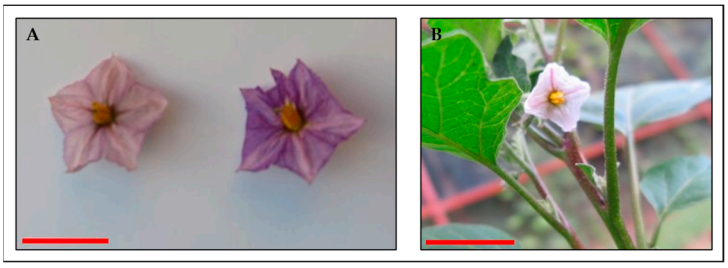


**Figure 6.** Variations observed in the flower morphologies of the mutant samples. Image (**A**) shows mutant 31-3 with a deeper hue of purple pigmentation (shown on the right), with controls shown on the left. Image (**B**) shows mutant 31-1 with reduced numbers of petals. Scale bar is 3 cm.

#### 3.3.5. Frequencies of the Observed Mutations among M2 Families

The frequencies of the analyzed variations in the M2 family are displayed in Table 5. The frequencies of the mutants were in the range of 14.3% to 28.6%. These observations of lower proportions of mutants imply the importance of screening multiple members of a mutant family to obtain a specific variation.

**Table 5.** Observed Mutations and Their Frequencies Across M2 Families.

| Observed Mutations | Total No. of Plants/Family | No. of Mutant Plants | Mutant's Frequency (%) |
|---|---|---|---|
| Dwarfism (9-1) | 6 | 1 | 16.7 |
| Leaf colour (36-5) | 7 | 2 | 28.6 |
| Curled leaf (39-6) | 7 | 1 | 14.3 |
| Glossy leaf (31-6) | 6 | 1 | 16.7 |
| Fruit shape (33-7) | 7 | 1 | 14.3 |
| Fruit shape (39-3) | 7 | 1 | 14.3 |
| Fruit color (18-2) | 6 | 1 | 16.7 |
| Flower color (31-3) | 6 | 1 | 16.7 |
| No of petals (31-1) | 6 | 1 | 16.7 |

Note: Numerical identifiers in brackets indicate sample identities.

## 4. Discussion

EMS mutagenesis was conducted on the Surya eggplant cultivar at 0.7% *v/v* of EMS concentration, and the resulting impacts were assessed through genotypic and visual phenotypic changes among the mutants. In the context of genotypic screening, we conducted

an initial evaluation of 16 individual lines across six targeted *FT*/*TFL1* genes using three biological control samples in a small-scale setting. This gene family is pivotal in the flowering regulation, as well as seed traits, of various crop species [31].

The genetic screening performed on the M2 mutants uncovered a heterozygous mutation in the predicted region of the 5′ UTR of *SmMFT*-2. The mutation was identified to be a T to C transition. EMS has been found to predominantly generate C-to-T substitutions, resulting in C/G to T/A transitions [34]. G/C to C/G or G/C to T/A transversions can also be induced at a low frequency through 7-ethylguanine hydrolysis, while A/T to G/C transitions are induced through 3-ethyladenine pairing errors [35]. However, whole genome profiling of EMS mutagenesis conducted on tomato revealed nearly equal proportions of A/T to G/C transitions, contradicting the reported bias for C/G to T/A transitions [36]. This study observed the same type of mutation, A/T to G/C transition, possibly owing to the potential chances of expecting them nearly equal to C/G to T/A substitutions. The impact of the mutation in the predicted 5′ UTR on flowering or seed traits has to be further validated. Since mutant populations could harbor genetic variations that may not manifest in observable characteristics, particularly when they occur as heterozygous recessive mutations [32], they could be further utilized in gene functional studies by recovering the mutation in a homozygous state in the next generation [33].

Despite the limited scope of this study in screening a small set of genes for random mutations induced by EMS, it successfully showcased the application of long amplicon sequencing using PacBio technology. Additionally, the subsequent analysis demonstrated the feasibility of detecting mutations and determining their zygosity, as pbLAA analysis has provided phased consensus sequences from pooled amplicons. Furthermore, comparable mutation detection approaches can also be beneficial for conducting forward genetic screening of the mutants acquired in this study, that exhibited various modified characteristics.

In addition to performing genotypic screening, a database was established to catalog the distinct variations identified within the M2 population. This initiative aimed to broaden the collection of valuable mutants available for future use. In this study, only the M2 generation was subjected to morphological screening, as it is not possible to detect mutations of recessive characters in the M1 generation, in which only mutations of dominant characteristics are observable [20]. Moreover, mutants of the M1 generation are chimeric, making it challenging and unreliable to detect them [37]. Consequently, the M2 generation is regarded as the optimal stage for screening mutants using forward or reversed methods [20].

The evaluation of the mutant population generated in this study showed that plant height was among the traits that exhibited a widespread impact of EMS mutagenesis. A previous analysis of eggplant reported comparable findings [20], in line with mutagenesis studies conducted on tomato [38] and soybean [39], perhaps because phenotypes with higher mutation rates are controlled by a larger number of structural and regulatory genes [20].

Among these mutants, one mutant (sample 9-1) displaying dwarfism was isolated. Dwarfism represents one of the most readily noticeable and common types of mutants. Dwarf mutant lines isolated from mutagenesis of various plant species have proven to be useful in adding to the cumulative knowledge pertaining to the genetics of plant growth and development [40]. Similarly, dwarf mutants discovered in eggplant could provide useful insights for understanding the regulatory mechanisms influencing the trait in the crop. Updates on monogenic, recessive mutations responsible for dwarfism could help to reveal novel genes underlying activities governing plant development [41].

A number of dwarf genes have shown close associations with hormone signaling pathways, such as gibberellins (GA), brassinosteroids (BRs), auxin (IAA) and/or strigolactones (SLs). For example, the tryptophan-deficient dwarf1 (*tdd1*) rice mutant exhibits embryonic lethality due to impaired organ development caused by the *TDD1* gene encoding a protein that functions upstream of Trp-dependent IAA biosynthesis. Another instance of a dwarf mutant in rice showed insensitivity to gibberellic acid (GA) and exhibited a

severe dwarf phenotype with elevated levels of endogenous GA. In *Arabidopsis*, the dwarf mutant shrink1-D (*shk1-D*) is caused by activation of the *CYP72C1* gene, a member of the cytochrome P450 monooxygenase family responsible for regulating brassinosteroid (BR) inactivation. The dwarf11 (*d11*) rice mutant has reduced seed length and is controlled by the *D11* gene, encoding a novel cytochrome P450 enzyme involved in BR biosynthesis. Meanwhile, the dwarf 53 (*d53*) rice mutant is governed by the *D53* gene, which encodes a substrate of the SCFD3 ubiquitination complex and acts as a repressor of strigolactone (SL) signaling [40]. Recently, a dwarf mutant of eggplant (dwf) was isolated from a mutant library induced by ethyl methanesulfonate (EMS). Through subsequent genetic analysis, it was discovered that the dwarf phenotype was associated with a single recessive gene. Employing bulked segregant analysis (BSA), a candidate gene, *SmCPR1*, which encodes cytochrome P450 reductases (CPR1), was successfully identified [42].

There were a number of variations observed pertaining to the leaf structures. In particular, mutants exhibiting changes in leaf color were obtained, consistent with the common findings in various EMS mutagenesis analyses. These variations in color are indicative of chlorophyll deficiency, as previously reported by studies that have found a correlation between leaf color variation and a significant change in chlorophyll content [43–45]. The presence of a chlorotic mutant has been suggested to be a reliable indicator for assessing genetic effects resulting from mutagenic treatments [46]. Moreover, mutants with changes in leaf coloration are highly valuable for identifying genes responsible for chlorophyll metabolism. There are several genes that have been found to be associated with the emergence of chlorotic mutants. Instances of chlorotic mutants with defects in the *ChlH*, *ChlD* or *ChlI* gene encoding subunits of Mg-chelatase have been observed in *Arabidopsis*, rice, barley and tea. Moreover, defects in genes such as *OsYGL1*, encoding a chlorophyll synthase, and the chloroplast-localized gene *OsYLC1* were also responsible for producing chlorotic mutants through their impacts on the biosynthesis of chlorophyll and alterations to its content [40].

Additionally, another type of altered leaf morphology was identified in the form of curled leaf structures with downward curling which were persistent throughout the plant growth. Curled-leaf mutants present a valuable genetic resource because of their potential to breed drought-tolerant cultivars since they have the ability to regulate leaf transpiration and improve drought tolerance. In addition, leaf curling has the potential to enhance light energy absorption and increase the rate of photosynthesis [47]. In contrast to the leaf structures observed for the recessive pepper *flc* mutant which curls during the day and flattens at night or under certain moisture conditions [48], the mutant generated in this study retained its phenotype all day. These discrepancies indicate the potential of the mutation obtained for further functional analysis, and it could be desirable in breeding programs.

Furthermore, we also observed a mutant plant that had acquired 'glossy' leaf structures. 'Glossy' leaves are often associated with cuticular wax accumulation (long-chain hydrocarbon compounds), which forms on the plant surfaces. One crucial role of cuticular waxes is to act as a defensive barrier against various environmental stresses, including drought. In certain instances, drought stress induces changes to the composition and elevates the levels of cuticular waxes in *Arabidopsis*, wheat and rice. Certain genes regulating the biosynthesis and transport of cuticular wax hold potential for enhancing drought tolerance [49]. The discovery of the mutant with 'glossy' leaf structures could therefore be beneficial in elucidating the role of cuticular wax accumulation in drought tolerance in eggplant. The mutants observed in this study also exhibited changes in fruit shapes, such as oblong, curved and indented shapes. Since the genetic regulations that control fruit orientation, shape and size are not yet understood, these mutants could represent genetic resources for studying the underlying mechanisms that contribute to fruit shape-related traits [47]. Over the past few decades, eggplant breeders have placed great emphasis on various eggplant characteristics in their breeding programs. As a result, traits such as fruit size, color and shape have been the primary objectives [50]. Fruit shape is crucial

concerning the market value of horticultural produce, and its alteration can result in the generation of new, as well as profitable, varieties that meet market demand [47].

The fruit color of eggplant is of commercial importance, and understanding the genetic factors that regulate the trait requires in-depth research. A previous report of eggplant highlighted the emergence of a white-colored fruit mutant from a purple-pigmented wild type by means of EMS mutagenesis. Further transcriptomic analysis of this fruit color mutant enhanced the understanding of anthocyanin biosynthesis in eggplant peels [51]. Intriguingly, the mutant population in this study showcased the discovery of fruit color mutants with strikingly enhanced purple pigmentation of the fruit peel (samples 46 and 18-2). These findings provide valuable means to gain more profound insights into the mechanisms regulating anthocyanin accumulation in eggplant. Apart from the fruit peel of eggplant, anthocyanin biosynthesis also governs the flower coloration [52]. As such, anthocyanin biosynthesis could be investigated in a broader sense during the course of plant development involving various organs. In line with this possibility, the mutagenesis undertaken in this study also led to the changes in flower coloration, in addition to other changes in flower structures. Overall, the mutant population established has also generated appealing resources to explore further regarding flower biology.

Additionally, the mutant sample, 46 displayed alterations in more than one trait, with changes in fruit shape, as well as peel coloration. This observation is consistent with prior studies of mutagenesis that also reported mutations affecting multiple traits. A report of eggplant mutagenesis revealed mutants with up to six phenotypic categories [20]. The occurrence of multiple phenotypes in mutants may be due to the pleiotropic effects of a single mutation or the simultaneous occurrence of multiple mutations [53]. Meanwhile, the mutants' frequencies observed across their respective families were rather low, supporting the common theory that mutations are mostly recessive. Therefore, it is recommended to phenotype 8-10 plants per family to identify a mutant phenotype [54].

It is important to highlight that the alterations in the mutant populations were specifically performed during the M2 generation, as it was considered an efficient time so that recessive traits could be recognized through homozygosity in the composition of the mutational base. Moreover, the inheritance of mutant phenotypes needs to be analyzed in the M3 or M4 generations due to potential hindrance by DNA self-repair mechanisms [20]. Since there could be considerable numbers of mutations segregating in mutant populations, new phenotypes might also arise in subsequent generations owing to gene interactions, including epistasis [55].

Moreover, saturation mutagenesis determines the efficiency of EMS mutagenesis and is correlated with the sample size of a mutant population, with large populations often targeted for this purpose [20]. Nevertheless, the sample size is often preferentially chosen by the experimenters, with M2 families screened ranging from hundreds to thousands [20,56]. Interestingly, even small-sized mutant libraries have the potential to successfully innovate a crop species [57,58], which can be solely attributed to the ability of EMS to cause genome-wide random lesions [32]. In line with this relationship, we have showcased the feasibility of obtaining EMS-induced variations in a preliminary, small-scale set up, which holds promise as valuable material for future functional analysis.

## 5. Conclusions

In conclusion, our mutant library holds potential as a valuable resource for innovating the eggplant germplasm. A small set of mutants were analyzed in a reverse genetic strategy for nucleotide changes in the *FT*/*TFL1* homologs, often associated with flowering regulation and seed traits [31]. A mutation that was found at the predicted 5′UTR region has to be further verified for its underlying impacts. Any potential changes may imply the possible location of cis-regulatory elements in the 5′UTR. Meanwhile, the emergence of attractive traits, such as dwarfism; unique leaf structures, such as 'glossy', curled and chlorotic leaves; enhanced pigmentation of the eggplant peel; and larger fruit sizes, as well as variations in flower color and structures, was facilitated by EMS-mediated genetic

variabilities. The unique characteristics obtained are promising avenues for investigations pertaining to genetic regulation of diverse traits of agronomic interest. Therefore, the discovery of these new germplasms could be directed toward further functional analysis through myriad genetics and genomics approaches, in addition to presenting choices for breeding in eggplant improvement programs.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijpb14030053/s1, Table S1: Sequences of primers used in the first round of PCR; Table S2: Sequences of primers used in the second round of PCR; Table S3: Primer combinations used in the second round of PCR across M2 mutants; Figure S1: The gene structure of *SmMFT*-2 as predicted using the Fgenesh gene prediction tool; Figure S2: Mutations identified in the upstream region of *SmMFT*-2 gene of mutant sample 31-3.

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