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Abstract. A novel Chili pepper G1M1 mutant was produced from chili pepper Genotype 1 induced with 0.01% ethyl methane sulfonate (EMS). The aim of this research is to evaluate the effect of EMS induced mutation to the sequence of capsaicin synthesis gene *KasI* and its expression. Genetic profile of *KasI* genes of mutant plants and controls were analyzed by sequencing method and the results aligned and compared with the sequence of *Capsicum annuum KasI* (KM037709.1) on the NCBI. Capsaicin content was measured from fruit at 30-35 DPA (day post anthesis) using spectrophotometry method (Å280 nm). The results of this study indicated that the capsaicin content of G1M1 mutant plant did not differ significantly with G1 control, but of G1M1 tended to have higher capsaicin content than control. The *KasI* gene of G1M1 mutant shows the presence of substitution at some sites which causes the replacement of amino acid composition. This may cause differences in the capsaicin content between plants.

1. Introduction

Chili pepper (*Capsicum frutescens* L.) is one of horticultural commodities that are widely used and developed in tropical countries, including in Indonesia. In the culinary field, chili pepper is usually used as a cooking spice to give spicy sensation. Chili pepper is also used in the medical and pharmaceutical fields because of its compounds that function as medicines for diabetes, obesity, cancer, etc. [1]. In addition, the compounds contained in Chilipepper are known to have antioxidant activity, anticancer, antibacterial, anti-inflammatory and others [2].

The cause of the spicy taste in chili pepper is the presence of capsaicinoid compounds [3]. The amount of capsaicin substances contained in chili pepper and other plants of the Capsicum genus were positively correlated with the level of spiciness (pungency). The more capsaicin substances produced, the higher the spiciness [3]. Each species and variety of chili plants has different capsaicin content and spiciness levels [4] due to genetic variability [5].

The large number uses ofchili pepper cause the demand to increase. However, this high demand is not supported by adequate supply because of the low production of chili, especially in times of water stress, whether deficient or excess. This condition triggers breeders to do chili plant breeding, among others by mutating. One method of plant breeding that can be done is by inducing mutations with chemical mutagens, such as ethyl methane sulfonate (EMS). EMS is known to cause point mutations

that occur randomly and not specifically on certain genes. The type of mutation produced is substitution from the GC base to AT with mutation intensity of 1/3000 kb [6].

Chili pepper (*C.frutescens* L.) G1 is one of the commercial chili varieties found in the city of Malang [7]. This chili pepper line is reported to have some superior morphological characters including having a large number of leaves and branches [8]. In the previous study, induced mutations were done on chili pepper G1 using EMS with concentration of 0.01%, 0.02% and 0.04% [9]. The results showed that at the application of 0.01% EMS concentration the best chili pepper mutant plant was achieved based on its morphology and physiological characters. The mutant (G1M1) showed an increase in the number of leaves and branches, as well as the content of capsaicin in the leaves [9].

One gene that plays a role in determining capsaicin content is the *3-oxoacyl-ACP (acyl carrier protein) synthase* (*KasI*) gene. This gene plays a role in the biosynthesis of capsaicin in the branched fatty acid pathway for the formation of 8-methyl-6-nonenoic acid precursors through chain cyclic reactions [9]. The *KasI* gene polymorphism between wild type and mutant *C.chinense* plants also reported to cause significant differences in capsaicin content in the fruit [10]. Genes mutation can be detected by analyzing genetic profiles using sequencing methods [10]. The polymorphism of *KasI* gene the sequence of the mutant G1M1 that aroused from EMS mutation have never been reported yet and so as its effect on the capsaicin content. So,this study investigated whether the EMS induced mutation has an effect on *KasI* gene sequence and on the physiological character governed by the gene.

2. Materials and methods

2.1. Seed Sowing and Planting

Chili pepperline G1 as control plant and second generation G1M1 mutant (F2) were sowed in a medium consists of a mixture of soil, husk, and compost (2: 1: 1). Chili pepper seedlings that already had4-5 leaves (aged ≥21 HST or ± 3 weeks) were moved into pots containing planting media with the same components as the sowing media components. Each pot contained one chili seedling. Watering was done if necessary, fertilizing once a week with organic fertilizer, and pest control was done with natural pesticides. Measurement of capsaicin content was carried out on 3 control G1 and 14 G1M1 mutant chili pepper plants.

2.2. Capsaicin Content Measurement

Raw chili fruit aged 30-35 Days After Anthesis (DAA) was taken as many as 3 pieces. Capsaicin extraction was done by grinding 0.5 grams of chili pepper using mortar and pestel. The paste then added with 5 mL of absolute ethanol. The homogenate obtained was filtered using filter paper which has been placed on top of flacon tube. The obtained filtrate was diluted 10 times using absolute ethanol. The absorbance was then measured using a spectrophotometer at a wavelength of 280 nm. The absorbance value obtained was used to calculate the capsaicin content based on the linear equation between the absorbance value and the concentration of standard capsaicin solution that has been known previously.

2.3. KasI GeneProfile Analysis

Genomic DNA was isolated from the leaves of control G1 chili pepper and G1M1 mutants using the modified CTAB method [11]. The resulting DNA was then dissolved in 50 µL TE buffer and then DNA isolates were stored in the freezer at a temperature of -20 ^{o}C until further analysis.

Genomic DNA was amplified with specific primers for the KasI gene (KasIF-CTCGTGCTGATGGACTTGGA and KasIR-AATGTTTCTTGCTCGGACTCTCT) with 964 bp product. The PCR mix solution had a total volume of 40 µl, which consisted of 14 µl sterile aquadest, 20 µl PCR mix, 2 µl forward primer (10 µmol), 2 µl reverse primer (10 µmol), and 2 µl DNA samples. The DNA amplification process was carried out in 35 cycles, hotstart at 95 0C for 5 minutes, denaturation at 95 ^{o}Cfor 60 seconds, annealing at 61.3 ^{o}C for 60 seconds, extension at 72 ^{o}C for 60 seconds, and post extension at temperature of 72 ^{o}C for 7 minutes. PCR products were electrophoresed

on agarose gel with a concentration of 1% at a voltage of 60 V for 45 minutes. Electrophoresis results were visualized with UV-Vis GelDoc transiluminator. The amplicon obtained was sequenced to determine the sequence of the *KasI* gene nucleotide base and detect mutations.

2.4. Data analysis

Data of capsaicin content were tested parametric statistic by T-independent test with a significance level of 0.05 or non-parametric statistics with Mann-Whitney test with a significance level of 0.05. Sequencing results were processed and aligned. Multiple alignment results from each sample were compared with the *Capsicum annuum3-oxoacyl- (acyl-carrier-protein) synthase* (*KasI*) gene sequence, *KasI*-a allele, complete cds (KM037709.1) from NCBI.

3. Results and Discussion

3.1. The Capsaicin Content of Chili pepper (C. frutescens L.) G1 Control and Mutant G1M1

The average capsaicin content between G1M1 and G1 mutant control groups showed no significant difference (Figure 1A). Variations in capsaicin content were actually shown by the mutant group G1M1(Figure 1B). G1M1/10 was a mutant plant that has the largest capsaicin content compared to other mutant plants, namely 10.17 ± 0.356 mg/gram F.W.

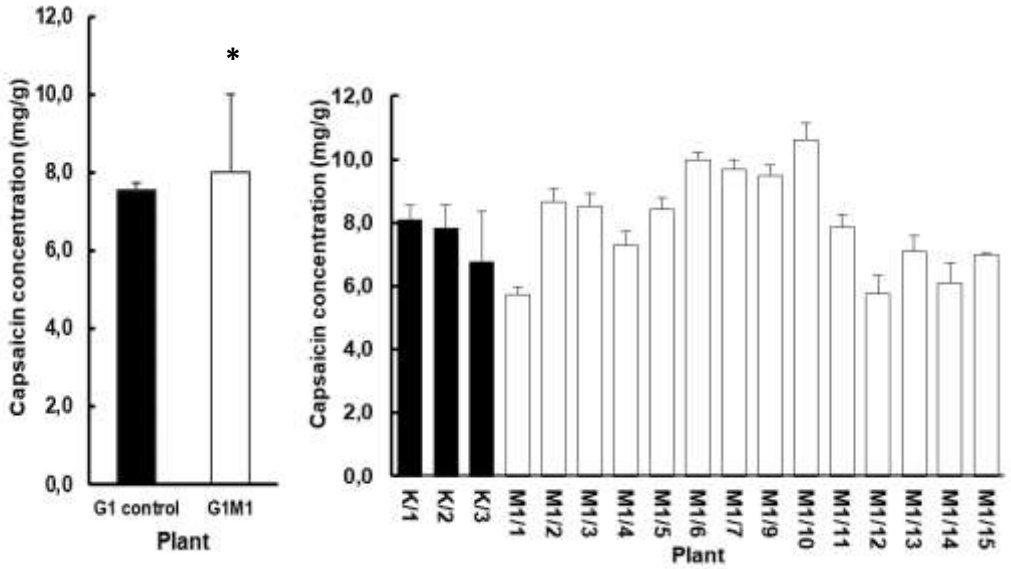

Figure 1. Capsaicin content of chilipepper (*C. frutescens* L.) G1 control and G1M1 mutant at 30 DAA. Average content (A); Between controls and mutants (B). Notation (*) indicates a significant difference between G1M1 plant groups and G1 control (n = 3) (α = 0.05).

Some studies stated that *C. frutescens* L. (4.24 ± 0.19 mg/gram F.W.) had higher capsaicin content compared to *C. annum* (1.38 ± 0.05 mg/gram F.W.) [5]. Chili spiciness level was classified into five groups: non-pungent (0-700 Scoville Heat Unit-SHU), mildly pungent (700-3000 SHU), moderately pungent (3000-25000 SHU), highly pungent (25000-770000 SHU) and very highly pungent (> 80000 SHU) [5, 12]. The SHU value can be used to predict capsaicin content by dividing the value of SHU with a conversion factor of 16.1 [13]. The results of the conversion of capsaicin content data to SHU values indicated that all mutant and control plants have very high capsaicin content (very highly pungent). Of all plant sample populations measured, capsaicin content from G1 controls was about 7 mg/gram, while mutant plants with the highest capsaicin content were around 10 mg/g and the lowest was around 5 mg/g. This large variation of capsaicin content was likely to occur because of the

random nature of EMS mutation induction causing mutations at different sites. Capsaicin content of chilli can be influenced by several factors including the expression of genes encoding enzymes or compounds that play a role in capsaicin biosynthesis [12].

3.2. Gen KasI profile on chili rawit (C. frutescens L.) G1 control and G1M1 mutants

The *KasI* gene is a gene that plays a role in the formation of the 3-oxoacyl-ACP (acyl carrier protein) synthetase enzyme which is responsible for the extension of the fatty acid chain during the biosynthesis process [8]. The whole *KasI* gene has a sequence length of 3456 bp, which is divided into 8 exons [14]. In this study the *KasI* gene was partially amplified to produce 964 bp (LG3) products. The results of the Basic Local Alignments Search Tool (BLAST) analysis between the sequence of the *KasI* gene in each plant with the sequence of *Capsicum annuum 3-oxoacyl- (acyl-carrier-protein) synthase* (*KasI*) gene, *KasI*-a allele, complete cds (accession code GeneBank KM037709.1) from NCBI showed high homology, which was 99%. Alligment results indicated several changes or differences in bases and some accompanied by changes in the amino acid composition of each sequence (Figure 2-3).

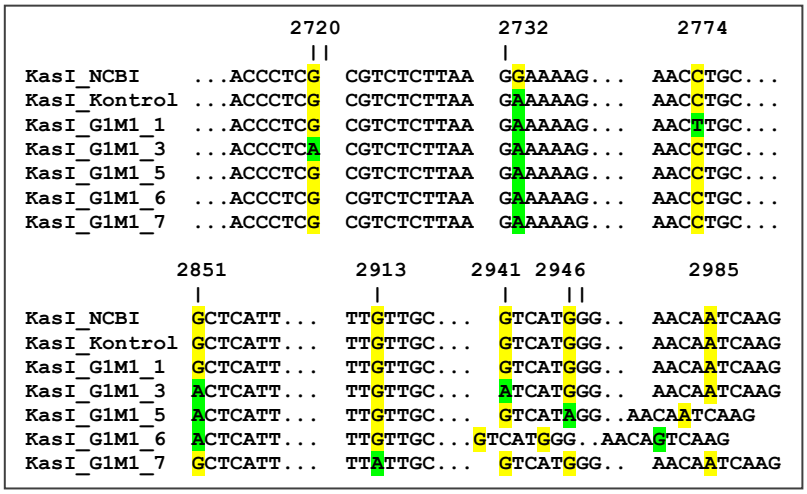

Figure 2. *KasI* gene profile ofchili pepper G1 control and mutant G1M1. Description: green higlight indicates a base change; yellow highlight shows a base that has not changed; (...) trimming sequence..

The control and mutant plants showed a base difference with the comparative *KasI* gene sequence from NCBI. The difference occurred because of substitution of guanine (G) to adenine (A) in base number 2732. This base change caused a change in the amino acid encoded which was glycine (GGA) to be glutamate (GAA). In the G1M1/1 plant there was one different base from the control *KasI* gene sequence, the difference was found in base number 2774, this difference occurs because of the cytosine substitution (C) to thymine (T), as a result the amino acids encoded also differ, namely proline (CCT) to leucine (CTT). In the G1M1/3 plant there were three base changes, namely substitution of guanine (G) to adenine (A) in bases number 2720, 2851, and 2941. These mutations cause changes in amino acids. At base number 2720, changes in the amino acid arginine (CGC) becomes histidine (CAC).At base number 2851, there was a change in amino acid alanine (GCT) to threonin (ACT). At base number 2941 there was a change in the amino acid valine (GTC) to isoleucine (ATC). In plant G1M1/5 there were two base changes, namely substitution of guanine (G) to adenine (A) in bases number 2851 and 2941. Both of these base changes cause changes in the amino acids formed at number 2851, alanine amino acids change (GCT) become threonin (ACT), and in the 2964 base number there was a change in the amino acid methionine (ATG) to isoleucine (ATA). In the G1M1/6 plant there were two base changes namely substitution of guanine (G) to adenine (A) in bases number 2851 and 2985. Mutations in base number 2851 cause changes in the amino acid alanine

(GCT) to threonin (ACT), while mutations in bases number 2985 does not cause changes in amino acids. In plant G1M1/7 there was one base change, namely substitution of guanine (G) to adenine (A) in base number 2913. This mutation does not cause changes in amino acids (Figure 10) (Figure 11) (Figure 2, 3). All base changes that occur were substituted base guanine (G) / cytosine (C) to adenine (A) / thymine (T) which iscommon in mutations due to EMS induction [6].

Comparing the sequence of the *KasI* gene between *C. annum* (KM037709.1) and G1 mutants and controls showed the presence of one different base and resulted in changes in one amino acid that changed, namely glycine to glutamate (Figure 3). This change in amino acids was probably one of the reason of differences in capsaicin content between *C. annum* and G1. Glutamate plays a role as enzyme cofactors branched chain amino acid amino transferase (BCAT)[3], this enzyme functions to convert glutamate to α-ketoglutarate (α-KG) [15]. α-ketoglutarate then acts as a substrate for the formation of acyl groups, the longer the C chain formed will affect the increase in chili spiciness [3]. Based on this, the possibility of being one of the causes of the large difference in capsaicin content between *C. annum* and G1 mutant and control plants was the difference in one amino acid in the partial sequence of this *KasI* gene.

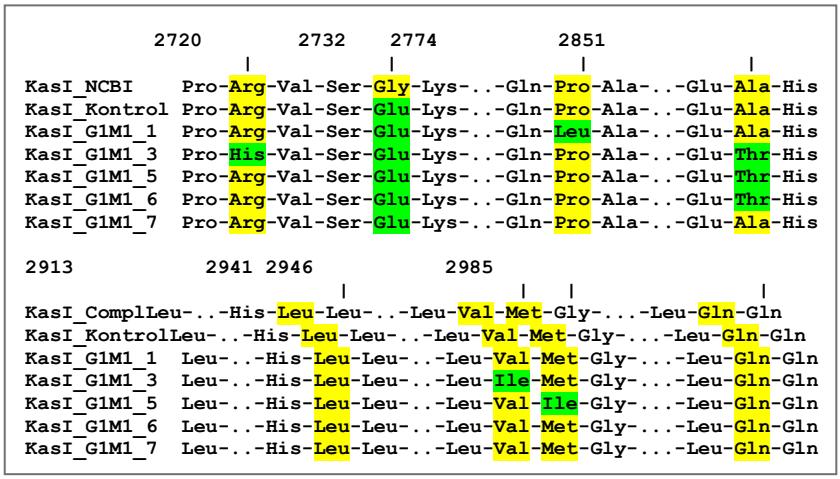

Figure 3. Amino acid profile chili pepper G1 control and mutant G1M1. Description: green higlight indicates a base change; yellow highlight shows a base that has not changed; (...) trimming amino acid sequence.

However changes that were observed on the nucleotides base and amino acids of the *KasI* gene did not affect the capsaicin content of the G1M1 mutant plant. This can be seen in G1M1/7 plants which did not have different amino acid composition with G1 control, but the capsaicin content was different. Plant G1M1/6 was known to be the plant that has the highest capsaicin content compared to other plants, based on the *KasI* gene sequence it can be seen that there was a change in amino acids compared to G1 control, namely alanine to threonine changes. Threonin was known as an amino acid that normally acts as a catalyst in branched-fatty acid biosynthesis in plants [15], so it was likely that with the presence of threonin, the fatty acid elongation reaction in the formation of capsaicin precursors becomes more optimal, resulting in more capsaicin. G1M1/3 plants were plants that have the largest differences compared with control plants, both morphologically and physiologically. *KasI* gene sequencing analysis on G1M1/3 plants also showed that this plant experienced the most base changes compared to the control and the *C.annum* from NCBI which was likely to be caused by EMS mutation.

4. Conclusion

EMS mutation changed the sequence of *KasI* gene resulted in alternation of amino acid produced. However, these changes did not correspond with the capsaicin content although all mutant plant to have higher capsaicin content than control. This phenomenon indicated that capsaicin content expression may be governed by many genes, not only KasI gene which may respons differently to the EMS induction.

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