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Journal of Multidisciplinary Applied Natural Science Vol. 5 No. 1 (2025) https://doi.org/10.47352/jmans.2774-3047.233
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Research Article

Screening of Sugarcane Commercial GMP Varieties Tolerant to Drought Stress Based on Molecular Detection of P5CS Gene

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Received : August 2, 2024	Revised : October 1, 2024	Accepted : October 24, 2024	Online : November 3, 2024

Abstract
Sugarcane (*Saccharum officinarum* L.) requires sufficient a water supply to produce optimal productivity. The selection of the superior varieties of sugarcane that are tolerant to drought can be done by molecular detection of the presence of the *pyrroline-5-carboxylate synthetase* (P5CS) gene. The research is aimed at molecular detection of P5CS gene in a commercial varieties at PT Gunung Madu Plantations (GMP), Indonesia that are potentially drought resistant. Similar research has never been conducted at PT GMP, as evidenced by the absence of official publications. The varieties used are GMP 3, GMP 5, RGM 06-654, PS 864, RGM 08-1026, PSJT 941, RGM 01-1834, GP11, RGM 07-099, and RGM 02-108. The research phases include DNA isolation, quantitative and qualitative DNA amplification, and PCR product visualization. Molecular data analysis is based on DNA band scoring results in the form of binary data which is then used to calculate the level of polimorphism information content (PIC) on the primary data used. The results of the study showed that nine out of ten varieties had P5CS-specific band sizes of ±167 bp, namely GMP 3, GMP 5, RGM 06-654, PS 864, PSJT 941, RGM 01-1834, GP 11, RGM 07-099, and RGM 02-108. A total of four of the nine varieties showed P5CS gene band thicknesses of RGM06-654, PSJT 941, PS864, and RGM 01-1834 which indicates the selected varieties most resistant to drought. The result of the PIC calculation has a value of > 0.25 which indicates the primary P5CS used is quite informative. The molecular detection was continued on one selected variety is PSJT 941, which showed that the relationship analysis of P5CS gene shows close affinity with the isolates from China and Bogor.

Keywords: molecular detection, P5CS gene, polymorphism information content, PSJT 941, commercial GMP varieties

1. INTRODUCTION

The limited availability of water that causes drought is a major limiting factor in crop production [1]. Sugarcane (*Saccharum officinarum* L.) is capable of responding to unfavourable environmental conditions to remain resistant to drought stress [2]. Adaptation as a response to drought stress can be studied on the basis of morphological characteristics [3][4], anatomy [5]-[10], physiology [11][12], and molecular [13][14]. The evolution of adaptation to dry stress is always based on molecularly complex mechanisms generated by a number of genes [15]. Several polypeptide-coding genes are supposed to play a role in protecting cells suffering from drought, including ion isolation, membrane stabilization, and accompanying molecules. One of the commonly used genes in screening drought-tolerant plants, whch plays a specific role in encoding the formation of proline is the pyrroline-5-carboxylate synthetase (P5CS) gene [16].

Prolin is one of the osmoprotective compounds that can protect plants from drought and excessive osmotic pressure [17]. The primary pathway of proline synthesis begins with glutamate which is converted to glutamic semi-aldehyde (GSA) by the P5CS enzyme. Subsequently, GSA is spontaneously converted into pyrroline-5-carboxylate (P5C) and P5C is then reduced to proline by the P5C reductase (P5CR) enzyme [15][17]. In addition to sugarcane, the P5CS gene has been found in several agricultural commodities, namely *Phaseolus vulgaris* [15], *Sorghum bicolor* [17], *Oryza sativa* [15][18], and *Triticum aestivum* [19]. Some reports suggest that plants that experience drought stress will carry out higher proline accumulation [20]. The prolin content of sugarcane stems treated with drought without irrigation for 15 d will rise almost 20 times [21].

PT Gunung Madu Plantations (GMP) is a pioneer Indonesian sugar company outside Java Island, namely in Lampung. Based on our previous research in 2021–2023, we have acquired eight varieties of local Lampung sugarcane that are

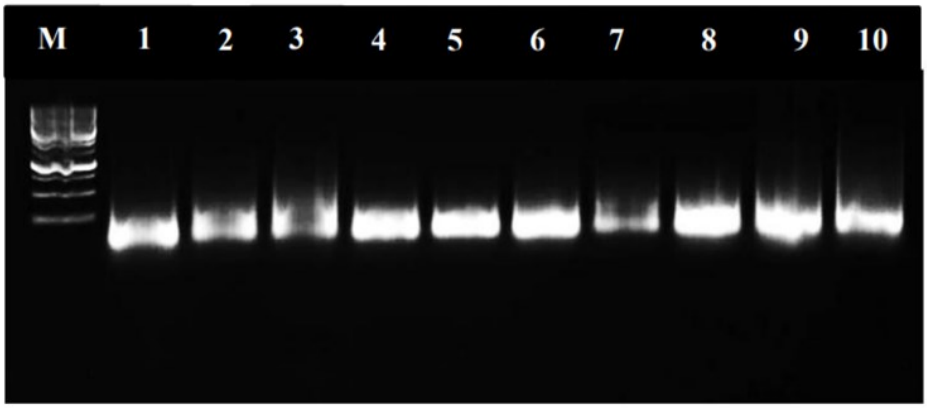

Figure 1. Visualization of DNA quality tests on 10 commercial GMP varieties. M: (Marker 1 kb), 1: GMP 3, 2: GMP 5, 3: RGM 06-654, 4: PS 864, 5: RGM 08-1026, 6: PSJT 941, 7: RGM 01-1834, 8: GP11, 9: RGM 07-099, 10: RGM 02-108.

potentially the ancestors of crop breeding [6][22]. GMP 1, GMP 2, GMP 3, GMP 4, GMP 5, GMP 6, and GMP 7 through a long selection process (10–12 years) according to the characteristics of the desired variety [23][24]. So far, 10 new commercial varieties have been obtained as a result of the breeding, namely GMP3, GMP5, RGM 06-654, PS 864, RGM 08-1026, PSJT 941, RGM 01-1834, GP11, RGM 07-099, and RGM 02-108, which have tolerant properties to droughts based on observations on the *in vitro* and greenhouse scales [6][25]. However, research on both yet fully validated the potentially superior traits of the sugarcane, thus further research is needed, specifically the molecular detection of the P5CS gene

In Indonesia, research on molecular detection of the P5CS gene in sugarcane is still limited. Prabowo et al. found P5CS gene identification results in 24 cultivars in PT Madu Baru, Bantul, Yogyakarta that showed a specific band of 167 bp [26]. Minarsih et al. conducted a partial cloning of the RT-PCR-derived P5CS gene marker from the PSJT 941 varietal that was cloned into *Escherichia coli* XL 1-Blue using the pGEM-TEasy plasmid vector [27]. The cloned DNA fragments were 984 bp of primary amplification P5CS_5F/ P5 CS_5R, 975 bp of primary magnification P5CS_3F/P 5 CS_3R, and 1725 bp of primary amplification P6CS_6F/R. The analysis showed that the sequence of the gene fragments had a very high homology (99%) with the P5CS genes in sugarcane and family plants. Based on this, the results of the molecular detection of the P5CS gene in 10 commercial GMP varieties that are tolerant to drought will help provide superior sugarcane seedlings and fulfil the national sugar demand.

2. MATERIALS AND METHODS

2.1. Materials

The sample is a six-month-old sugarcane leaves that have been grown in a greenhouse with drought resistant treatment. The 10 newly crossed commercial varieties of GMP are GM 03, GM 05, RGM 06-654, PS 864, RGM 1834, PSJT 941, RGM 1834 and RGM 944; RGM 08-1026, GP 11, RGM 07-099, and RGM 02-108. The sample collection following the previous methods [25][28][29].

2.2. Methods

2.2.1. DNA Extraction, Qualification, and Quantification

The initial stages are genomic DNA isolation, quantitative and qualitative testing, amplification, and electrophoresis. The protocol for the isolation of DNA using the method of cetyltrimethylammonium bromide (CTAB) [13] [29]. The principle of DNA isolation is divided into five stages: cell isolation, cell wall and membrane smoothing, solution extraction, purification, and presipitation [15]. The final result of the isolation is a DNA pellet that is added to 50 µL of 1X TE solution and stored at a temperature of –20 °C until DNA is used. DNA purity is tested quantitatively

using a spectroscopic photometer at 260 and 280 nm wavelengths [1], while quality testing uses electrophoresis with 1% agarose gel and 70 V voltage for 40 min [15][16]. The value of DNA purity ranges between 1.8–2.0. A DNA purity of less than 1.8 suggests contamination from protein, while a purity of more than 2.0 suggests contamination from chloroform and phenol [29].

2.2.2. Detection of P5CS Gene

Amplification of the P5CS gene was carried out using a specific primary pair following the method of Aristya et al. [30], namely P5CS-F 5'ACAGATGATGATTAAAGTAGCAGAGAC3' and P5CS-R 5'AGACCTTCAACACCCACAG3' with a product size of ±167 bp. The PCR composition was carried out in a volume of 25 µL, consisting of 1 µL primary F and primary R respectively, 12.5 µL PCR Mix, 8.5 µL ddH_2O, and 2 µL DNA sample. DNA amplification was carried out using a T100 thermal cycler (BioRAD). The initial denaturation was carried out at a temperature of 94 °C for 60 sec, followed by 40 cycles consisting of desaturation at a temperature of 94°C for 45 sec, annealing at 62 ° C for 60 sec, extension at a temperature of 72 °C For 75 sec, and final extension also at 72 ° C For 75 sec. Visualization of PCR products was conducted by using 2% agarose gel electrophoresis for 90 min at 100 Volts [30].

The DNA band is scored based on its appearance and is transformed into a binary data code in such a way that if there is a polimorphism information content (PIC) value scored one (1) and no band scored zero (0) [13]. The PIC values were used to determine the informative level of the markers used and were divided into three categories i.e., PIC > 0.60 (highly informative), PIC> 0.30–0.59 (quite informative) and PIC < 0.3 (less informative) [29].

2.2.3. Sequence Analysis of P5CS Gene

Nucleotide sequences were assembled using EditSeq and SeqMan from DNASTAR Lasergene DM Version 3.0.25. Homology analysis using the basic local alignment search tool (BLAST) program on the National Center for Biotechnology Information website (NCBI). Algorithm Multiple Alignment Parameters DNA using Kimura-2 parameters, Neighbor-Joining of MEGA 12 Beta, and relationship and phylogenetic analysis were used to analyze the sequences. The internal branch was statistically examined using the bootstrap values with 1000 replications [14].

3. RESULTS AND DISCUSSIONS

3.1. DNA Qualification and Quantification

The DNA qualitative test results using 1% electrophoresis gels showed a whole variety of tested sugarcanes showing genome DNA visualizations that seemed intact (Figure 1). The absence of a DNA smear during electrophoresis characterizes visibly intact genome DNA. Saraswathi and Mullainathan explained that this is important because in further molecular analysis, genome DNA that is still intact will give more accurate results [31]. Each sample's quantitative

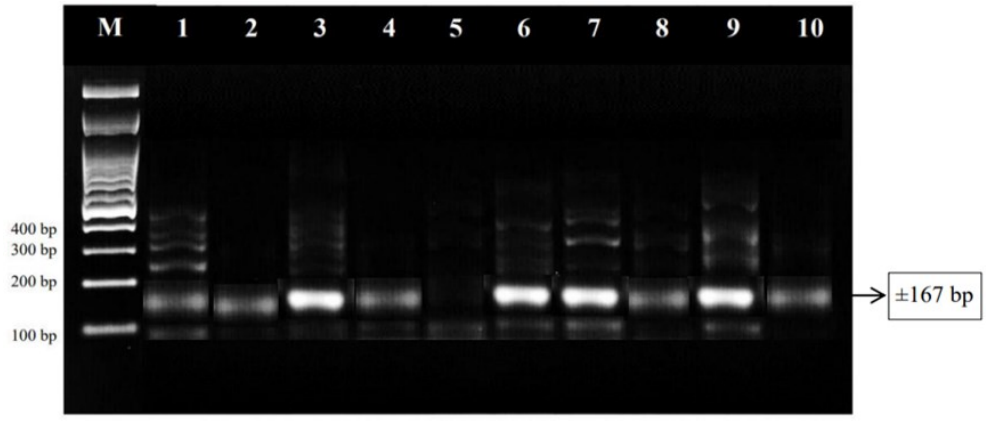

Figure 2. Visualization of P5CS gene amplification results on 10 commercial GMP varieties. M: (Marker 1 kb), 1: GMP 3, 2: GMP 5, 3: RGM 06-654, 4: PS 864, 5: RGM 08-1026, 6: PSJT 941, 7: RGM 01-1834, 8: GP11, 9: RGM 07-099, 10: RGM 02-108.

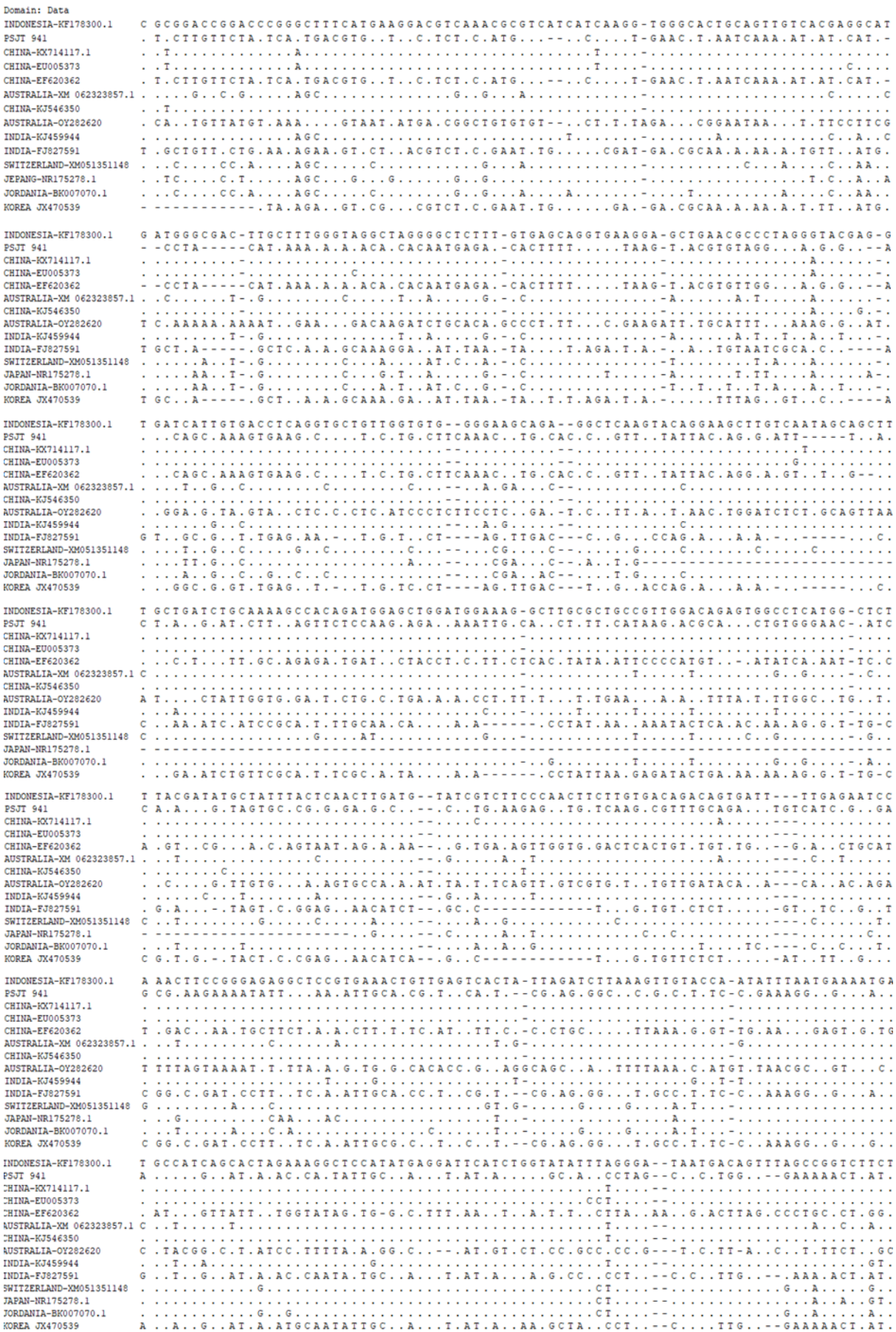

Figure 3. Alignment analysis of 8 sequence nucleotides showing sample mutations.

Table 1. Frequency of amino acid content in PSJT 941Lampung isolate.

Frequency of amino acid (%)																			Total
Ala	Cys	Asp	Glu	Phe	Gly	His	Ile	Lys	Leu	Met	Asn	Pro	Gln	Arg	Ser	Thr	Val	Trp	Tyr	
2.06	3.09	2.06	3.09	5.15	2.06	3.09	8.25	4.12	8.25	5.15	7.22	2.06	3.09	11.34	7.22	10.31	8.25	1.03	3.09	**97**

DNA test results revealed a purity range of 1.8–1.9. This indicates that all samples have a level of DNA purity that meets the established standard which is in the range of 1.8–2.0 values [13]. GP 11 has the lowest absorption value at 1.82, while RGM 07-099 has the highest absorbance at 1.96. According to Aguilar et al., the values were still in the standard range indicating the absence of oxidizing components like phenols, salts, proteins, or other oxidative molecules that absorb at 280 nm [32].

Based on the DNA concentration of the genomes, the isolation results for each sample still have good DNA purity. The lowest DNA concentration is in GP 11 varieties at 637 ng/µL, while the highest is in RGM varieties 07-099 at 1,835 ng/µL. Vaze et al. explained that one of the factors affecting the concentration of genome DNA is that the selection of leaf tissue samples in DNA isolation also affects the amount of DNA obtained [33]. Low DNA concentrations can be caused by the accumulation of phenolic compounds, too-old leaf samples, or DNA damage due to extraction treatment [34].

3.2. Detection of P5CS Gene

DNA amplification results on all samples showed specific bands of ±167 bp in nine commercial GMP varieties, namely GMP 3, GMP 5, RGM 06-654, PS 864, PSJT 941, RGM 01-1834, GP 11, RGM 07-099, and RGM 02-108. While RGM 08-1026 varieties did not visualize DNA bands. A detailed visualization of P5CS gene amplification results in 10 commercial GMP varieties is shown in Figure 2.

The appearance of DNA bands with lengths corresponding to the size of the target genes and parallel bands between the varieties suggests that the nine sugarcane varieties are superior varieties that may be tolerant to drought [35]. The visualizations of DNA bands also exhibit varying thicknesses, with four varieties, namely RGM 06-654, PSJT 941, PS 864, and RGM 01-1834, displaying a thicker and brighter DNA band than others. This suggests that these four varieties are most likely to be resistant to drought. According to Kumar et al. [2], drought-resistant varieties with thick and accumulated DNA band profiles exhibit high and intact target gene concentrations [36].

The calculated PIC value for the entire sample is

0.46. This indicates that the P5CS primer used is quite informative as a molecular marker because it has a PIC value >0.25. According to Medeiros et al., the utilized primary aligns well and merits use as a molecular marker [37]. The DNA band's ability to produce the result of a PCR reaction using a specific primer is the primary informative level of DNA. The range of genotypes used Amiteye influences the informative degree [38], which heavily depends on the primary recognition of its complementary DNA sequence on the used DNA template [39][40]. The informative level of the DNA marker can be assessed by looking at the PIC value. The PIC value represents the production power of a DNA band produced by a PCR reaction using a specific primer. A higher PIC value indicates the production of more informative DNA band patterns [41]. PIC quantification measures the number of alleles a marker can produce and the frequency of each allele in a set of tested genotypes. The frequency of allele appearance determines the value of polymorphism [2].

3.3. Expression of P5CS Gene

Analysis of the P5CS gene sequence in PSJT 941 from Lampung, Indonesia, showed a 401 base sequence with a base content percentage of T(U) 27.9%, C 18.8%, A 31.0%, and G 22.3%. This indicates a GC content (41.1%) that is slightly lower than the AT(U) content (58.9%). Hu et al. proposed that genomes with GC-rich content are more adapted to high environmental changes, as GC pairs are generally more stable than AT pairs [42]. A homologous sequence-sequence search using BLAST in NCBI was conducted to ensure that the test sample is a P5CS gene in *S. officinarum*. The results of homological analysis showed that the P5CS gene isolate in *S. officinarum* originating in Lampung, Indonesia, had similarities with Chinese (KX714117, EU005373, EF620362, KJ546350), Australia (XM_0623857, OY282620), Bogor, Indonesia (KF178300), India (KJ459944, FJ827591), Switzerland (XM_051351148), Japan (NR_175278), and Africa (X M_010932898) with the highest similarity index percentage of 99.51%. This suggests that the entire isolate has a close affinity based on low genetic distance values. According to Johnson et al., the level of genomic sequence homology between isolates with > 95% identity represents the same genus, whereas sequences with > 97% identity represent the same species [43].

The nucleotide alignment analysis with 13 other isolates and outgroups (Figure 3) revealed differences in the P5CS gene nucleotides between the PSJT 941 isolate and the other isolate with mutations. The mutation analysis showed 30 delays, 6 inserts, 29 transversions, and 35 transitions. Iengar explains the presence of multiple long insertion, deletion, and substitution mutations in the genome caused by adaptation to changes in the biotic and abiotic environments [44].

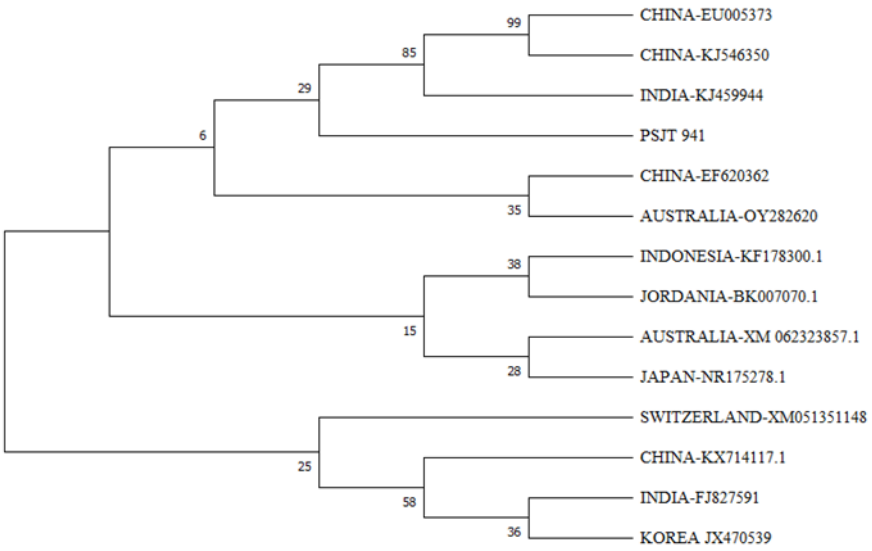

Figure 4. Phylogenetic tree on isolated PSJT 941 Lampung with 1000 boostraps.

Mutations will always lead to changes in the sequence of synthesized amino acids [45]. The analysis of amino acid frequency in PSJT 941 isolates (Table 1) revealed that the occurrence of mutations led to changes in amino acids in both the isolates and other isolates. Based on the changes in the sequence of amino acids and the frequencies of amino acids in the isolates, the test showed a higher amino acid content with a total amino acid content of 97%. Johnson et al. reported similar results, indicating that the levels of similar amino acid sequences in the same group ranged between 83–99% and 39–53% in different groups [43]. It is also consistent with the analysis of genetic distance values, which show that the test isolates have very different genetic range values from other isolates in the range of 0.004–2.017. Kaehler et al. explained that genetic distance measures the number of changes that distinguish biological sequences and is a fundamental metric in molecular evolution [46]. Genetic distances based on consistent nucleotide sequences are always greater than those based on amino acids.

The phylogenetic tree reconstruction shows that the P5CS gene isolate from Lampung is different from other isolates (Figure 4). The isolates continue to exhibit strong similarities with isolated Chinese origins (EU005373, KJ546350) and India (KJ459944). The dendrogram shows that the isolates are still in the same branch as other isolates, namely Bogor, Indonesia (KF178300), Australia, Jordan, and Japan. Though they come from the same country as Indonesia, Isolate test and Bogor have no close relations. The test isolate PSJT 941, a wildtype variety, serves as a cross-crossing parent, whereas the Bogor isolate is a cross hybrid. Xiong et al. explained that cross-produced hybrids have a low genetic variation, so they have distant affinities with wild-type varieties [47]. The limited introgression in sugarcane has resulted in a narrow genetic base for the current commercial varieties. Therefore, in sugarcane breeding, it is still a critical task to broaden the genetic base of sugarcane crops and improve environmental stress resistance using the gene pool of wild relatives.

In addition to the influence of human activity that causes a decrease in genetic variation due to breeding, the grouping of these isolates is due to the relatively close geographical area, so that the proximity between one isolate and the other is isolated. The phylogenetic tree reconstruction results indicate that the number of mutations, which have so far led to the formation of varieties up to new species, is what groups the isolates together. Shapiro et al. [48] and Schierenbeck [49] explain that the factors leading to the formation of new varieties and species are the evolution of diversity, the introduction of host genes that are susceptible to intersectional processes, and climate change [50].

4. CONCLUSIONS

A total of nine out of 10 commercial GMP varieties at P PT Gunung Madu Plantations express the specific P5CS gene band of ±167 bp, namely GMP 3, GMP 5, RGM 06-654, PS 864, PSJT 941, RGM 1834, GP 11, RGM 07-099, and RGM 02-108. Varieties of RGM 06-654, PSJT 941, PS 864, and RGM 01-1834 showed visualization of the brighter gene band thickness, potentially as a more resistant to drought varieties. The result of the calculation of the PIC value has a value > 0.25 which indicates the primary P5CS used is quite informative. Analysis of P5CS gene expression in the PSJT 941 variety showed close affinities with Chinese and Bogor isolates.

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Conflicts of Interest

The authors declare no conflict of interest.

ACKNOWLEDGEMENT

The authors would like to appreciate the funding assistance provided by PT Gunung Madu Plantations (GMP) for the research collaboration, grant numbers 023-00/GMP/I/2021, 013-00/GMP/I/2022, and 025-00/GMP/I/2023.

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