HAYATI
Journal of Biosciences

Vol. 33 No. 3, May 2026 575-583
DOI:10.4308/hjb.33.3.575-583
ISSN: 1978-3019
EISSN: 2086-4094

Research Article

Characterization of Lignin Biosynthesis Encoding Gene, *SiCOMT*, from Nine Indonesian Foxtail Millet (*Setaria italica* (L.) P. Beauv.) Genotypes

Ramadaniarto Rizqullah[1], Nirwanti Saenal[2], M Reza Pahlevi[1], Willy Bayuardi Suwarno[3], Daisuke Tsugama[4], Sintho Wahyuning Ardie[3]*

[1]*Plant Breeding and Biotechnology Study Program, IPB University, Bogor 16680, Indonesia*
[2]*Agronomy and Horticulture Study Program, IPB University, Bogor 16680, Indonesia*
[3]*Department of Agronomy and Horticulture, IPB University, Bogor 16680, Indonesia*
[4]*Asian Research Center for Bioresource and Environmental Sciences (ARC-BRES), Graduate School of Agricultural and Life Sciences, The University of Tokyo, Japan*

ARTICLE INFO

Article history:
Received September 19, 2025
Received in revised form December 7, 2025
Accepted December 8, 2025
Available Online December 23, 2025

KEYWORDS:
Feed,
methyltransferase,
protein structure prediction,
SNP,
structural stability

ABSTRACT

Caffeic acid O-methyltransferase (COMT) is an essential enzyme that catalyzes the biosynthesis of lignin monomer units. Foxtail millet (*Setaria italica*) possesses three COMT-encoding genes. However, only *SiCOMT1* and *SiCOMT2* are considered expressed. This study investigates the characteristics of the two COMT-encoding genes across nine Indonesian foxtail millet genotypes. Phylogenetic analysis revealed that *SiCOMT1* is closely related to N-methyltransferase genes, which are not involved in the lignin biosynthesis pathway. Meanwhile, *SiCOMT2* is closely related to O-methyltransferase genes involved in lignin biosynthesis. *SiCOMT2* from nine Indonesian foxtail millet genotypes exhibits 15 synonymous and three non-synonymous SNPs. *SiCOMT2* amino acid showed Ala67Thr and Pro72Ala variations within the methyltransferase dimerization domain, and Glu146Asp within the O-methyltransferase domain. Among these, the Pro72Ala substitution is predicted to reduce the structural stability of the encoded protein. These findings suggest that *SiCOMT2* may serve as a promising target for future genetic research and crop improvement strategies aimed at enhancing biomass quality by modifying lignin content and composition.

1. Introduction

Foxtail millet (*Setaria italica* (L) P. Beauv.) has been reported to exhibit strong adaptability to climate change and tolerance to abiotic stress (Ratnawati *et al.* 2024; Ardie *et al.* 2025), making it a suitable crop for cultivation in marginal areas (Aribam *et al.* 2024). The grain is also considered a potential functional food, offering high nutritional value similar to rice, with a higher protein content (Arora *et al.* 2023; Kalsi and Bhasin 2023). Additionally, foxtail millet can also be utilized as animal feed. Foxtail millet grain can increase broiler chickens' weight and protein content (Boroojeni *et al.* 2011), while the forage can be processed into silage for ruminants' feed (Topçu *et al.* 2025). However, the use of foxtail millet as forage for animal feed requires careful consideration of its lignin content, which is one of the factors determining feed quality and can negatively impact digestibility and overall feed efficiency (Zhong *et al.* 2021; Rahman *et al.* 2025).

*Corresponding Author
E-mail Address: sintho_wa@apps.ipb.ac.id

Lignin is a major component of plant cell walls, contributing to structural integrity and resistance to environmental stress (Yadav and Chattopadhyay 2023). It is a complex polymer composed of three primary monolignols: hydroxyphenyl (H unit), guaiacyl (G unit), and syringyl (S unit) (Sulis *et al.* 2025). Lignin biosynthesis involves multiple enzymes, including caffeic acid O-methyltransferase (COMT; EC 2.1.1.68). COMT catalyzes the methylation of 5-hydroxyconiferaldehyde and 5-hydroxyconiferyl alcohol to produce sinapyl alcohol, the precursor of the S unit (Peracchi *et al.* 2024).

The S unit has been reported to influence cell wall digestibility. A moderate lignin content, combined with a high S/G ratio, has been shown to enhance the delignification process and improve digestibility (Vanhevel *et al*. 2024; Yusron *et al*. 2024). Disruption of COMT enzyme activity reduces the accumulation of S units, thereby lowering the S/G ratio and ultimately decreasing cell wall digestibility (Fornalé *et al.* 2015). These findings highlight the critical role of the *COMT* gene in determining forage digestibility for animal feed.

Muthamilarasan *et al.* (2015) reported that foxtail millet possessed three COMT enzyme-encoding genes, namely *SiCOMT1, SiCOMT2*, and *SiCOMT3*. However, only *SiCOMT1* and *SiCOMT2* were highly expressed, whereas *SiCOMT3* expression was low across all plant tissues. Thus, this study aims to characterize the *SiCOMT1* and *SiCOMT2* genes from Indonesian foxtail millet, identify the gene variations, and predict the nucleotide base variation effect on the protein activity stability.

2. Materials and Methods

2.1. Genetic Materials Preparation

The research was conducted at the Plant Molecular Biology 2 Laboratory, Department of Agronomy and Horticulture, Faculty of Agriculture, IPB University, Indonesia. The DNA genome was extracted using the CTAB method (Aboul-Maaty and Oraby 2019), with slight modifications in the buffer composition. The modified CTAB buffer consisted of 3% (w/v) CTAB, 0.5 M Tris-HCl pH 8, 1.4 M NaCl, 0.02 M EDTA pH 8, and 3% (w/v) polyvinylpyrrolidone (PVP). We used foxtail millet leaves at the early-growing-stage seedling stage (about 3-4 weeks after sowing) to isolate DNA from the following genotypes: Botok4, Botok10, Buru, Hambapraing, Mauliru2, ICERI-5, ICERI-6, NTB1, and Toraja. The DNA concentration and quality ($A_{260}/_{280}$ and $A_{260}/_{230}$ ratios) were estimated using a spectrophotometer (MN-917 Maestro Nano, Taiwan).

2.2. DNA Amplification and Sequencing

Gene-specific primers were designed to amplify the *SiCOMT1* and *SiCOMT2* genes based on the sequence reported by Muthamilarasan *et al.* (2015). The target genes were systematically divided into multiple overlapping fragments to ensure complete coverage of the coding regions. Each fragment was subsequently amplified by PCR using the specific primer pairs detailed in Table 1, with the primer positions illustrated in Figure 1A for the *SiCOMT1* gene and Figure 1B for the *SiCOMT2* gene. The overlapping PCR strategy, utilizing multiple primer pairs to generate partially overlapping fragments, was employed to overcome

Table 1. Primer list used for *SiCOMT* gene amplification

Primer name	Nucleotide base sequence (5'-3')	Ta (°C)	Amplicon range (gDNA)	Amplicon size (bp)
SiCOMT1-Fw1	CCAATTTCCATCTCGTCCCG	63	1-709	709
SiCOMT1-Rv1	AGGTCGAAGTTGATCCCCTC			
SiCOMT1-Fw2	GCCAACGAGGTGATGCTC	63	522-1,271	750
SiCOMT1-Rv2	AACGGCAGTGGAGGAGTA			
SiCOMT1-Fw3	TACTCTGTCCGTTCTGGTTT	63	971-1,700	730
SiCOMT1-Rv3	GTTGCGGAACTCGTCCTC			
SiCOMT1-Fw4	CTAATGGCAGTGGGTCCT	58	1,472-2,126	655
SiCOMT1-Rv4	GTGCGAGTGAATCAAAACC			
SiCOMT2-Fw1	CTGAGCACACGCCACCAC	63	85-1,096	1,011
SiCOMT2-Rv1	CTCAGTCCATAACAGCACGG			
SiCOMT2-Fw2	GTAAGCAGGCGCCAAAGTTGT	63	781-1,440	660
SiCOMT2-Rv2	AGAACAGCGTCCTTCAGGTA			
SiCOMT2-Fw3	ACTATGGCAGTACACCCCAA	63	1,228-2,089	862
SiCOMT2-Rv3	TCACTTGGTGAACTCGATGG			

Ta: annealing temperature, gDNA: genomic DNA, bp: base pairs

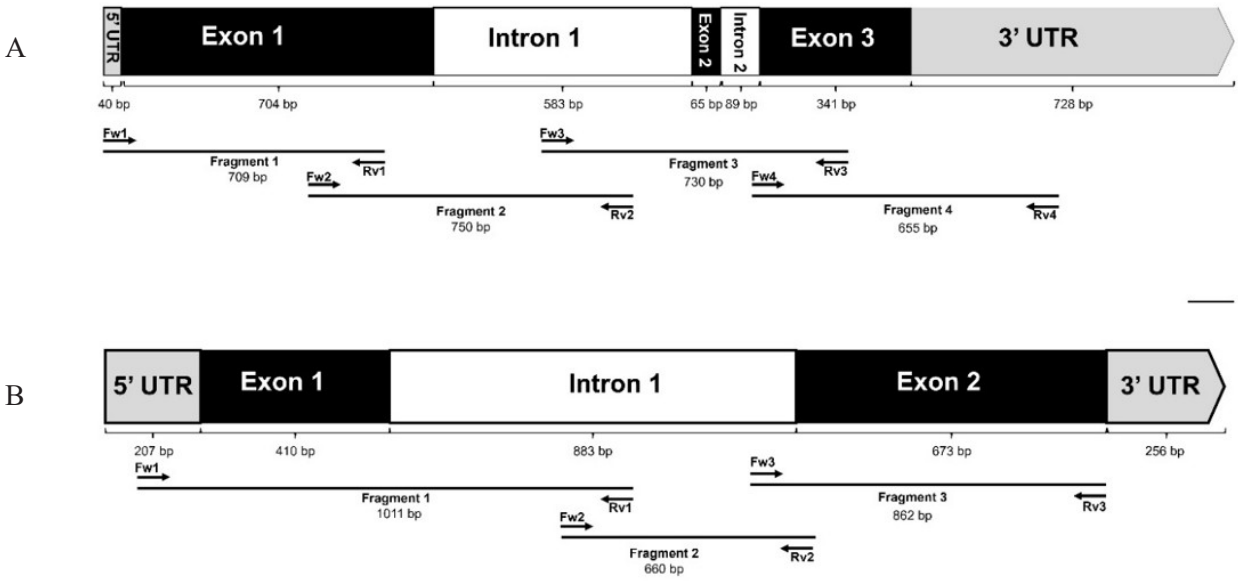

Figure 1. (A) *SiCOMT1* and (B) *SiCOMT2* genes forward (Fw) and reverse (Rv) primer scheme from genomic DNA (gDNA) of foxtail millet

technical limitations in long-fragment amplification. This technique facilitated reliable amplification, proper primer design, and accurate reconstruction of the full sequence by Sanger sequencing.

Gene fragments derived from nine foxtail millet genotypes were amplified using a thermal cycler (Bio-Rad T100; Bio-Rad Laboratories, USA). Each PCR reaction was prepared in a total volume of 50 µL, containing 200 ng of genomic DNA as template, gene-specific forward and reverse primers (0.2 µM each), MyTaq HS RedMix polymerase (1× final concentration), and nuclease-free water. Amplification was conducted under the following cycling conditions: an initial denaturation at 94°C for 1 min, followed by 35 cycles consisting of denaturation at 94°C for 15 s, primer annealing at 63°C for 15 s, and extension at 72°C for 10 s. However, for fragment 3 of the *SiCOMT1* gene, amplification was performed with an annealing temperature of 58°C and 45 cycles. A final extension step was performed at 72°C for 10 min to ensure complete synthesis of the amplified products.

The amplified PCR products were analyzed with electrophoresis on 1.5% (w/v) agarose gel at 90 V for 45 minutes in 1 × TAE (Tris-Acetate-EDTA) buffer, then stained using ethidium bromide (0.5 µg/mL), followed by visualization using a UV transilluminator (Gel Doc EZTM, Bio-Rad, USA). The visual representation of the PCR amplification results is provided in the Supplementary File. The unpurified PCR products with the expected band size were sent to 1st BASE (Singapore) for bidirectional Sanger DNA sequencing.

2.3. Data Analysis

The *SiCOMT1* and *SiCOMT2* genes (Gene IDs: *Seita.1G72500* and *Seita.6G055900*), as reported by Muthamilarasan *et al.* (2015), were queried against the NCBI BLAST database to identify homologous sequences from the whole-genome assembly of *Setaria italica* cv. Yugu1 (GCA_000263155.2). These homologous sequences were then used as reference sequences for alignment with the *SiCOMT1* and *SiCOMT2* sequences from Indonesian foxtail millet genotypes. The alignment analysis was performed using Bioedit 7.2 software with the MUSCLE alignment algorithm (Edgar 2004).

The phylogenetic tree was constructed using the maximum likelihood method in IQ-TREE, with 10,000 bootstrap replicates. The analysis utilized the transversional model (TVM), incorporating empirical base frequencies (F) and gamma-distributed rate heterogeneity across four discrete categories (G4) (Trifinopoulos *et al.* 2016), incorporating 17 homologous genes from other plant species retrieved from the NCBI database (Supplementary Table S1), then visualized using iTOL (Letunic and Bork 2024). The aligned translated amino acid of *SiCOMT* genes were used for protein structure analysis using Phyre2.2 (Kelley *et al.* 2015; Powell *et al.* 2025) and AlphaFold2 (Jumper *et al.* 2021). Change in folding energy value

(ΔΔG) prediction analysis was also performed using I-Mutant2.0 (Capriotti *et al.* 2005) with the following parameters: 25°C temperature and pH 5.48 estimated using Expasy ProtParam (Gasteiger *et al.* 2005). Structural superimposition was conducted in PyMOL v3.1.3 using the 'align' command. The predicted protein model was fitted onto the reference structure to enable quantitative comparison of the overall fold and the spatial arrangement of residues.

3. Results

The *SiCOMT1* and *SiCOMT2* genes were successfully amplified from nine Indonesian foxtail millet genotypes. Phylogenetic analysis based on coding DNA sequences (CDS) revealed that the *SiCOMT1* and *SiCOMT2* genes were grouped into distinct clades (Figure 2). The *SiCOMT1* gene from nine Indonesian foxtail millet genotypes clustered within clade I, together with genes encoding N-methyltransferase, known to be uninvolved in the lignin biosynthesis pathway, from other plant species. Conversely, the *SiCOMT2* coding sequences (CDS) from nine Indonesian foxtail millet genotypes clustered within group II, alongside COMT genes from other plant species classified as O-methyltransferases. Considering that *SiCOMT1* may not contribute to lignin biosynthesis, further analyses focused solely on *SiCOMT2*.

Alignment of *SiCOMT2* sequences from nine Indonesian foxtail millet genotypes with the reference sequence revealed a total of 13 single-nucleotide polymorphisms (SNPs) in the intron (Figure 3A) and five SNPs in the exon (Figure 3B). A nucleotide substitution at position 1,423 (A→T) within intron 1 may disrupt the splicing branch point. Non-synonymous SNPs were identified in

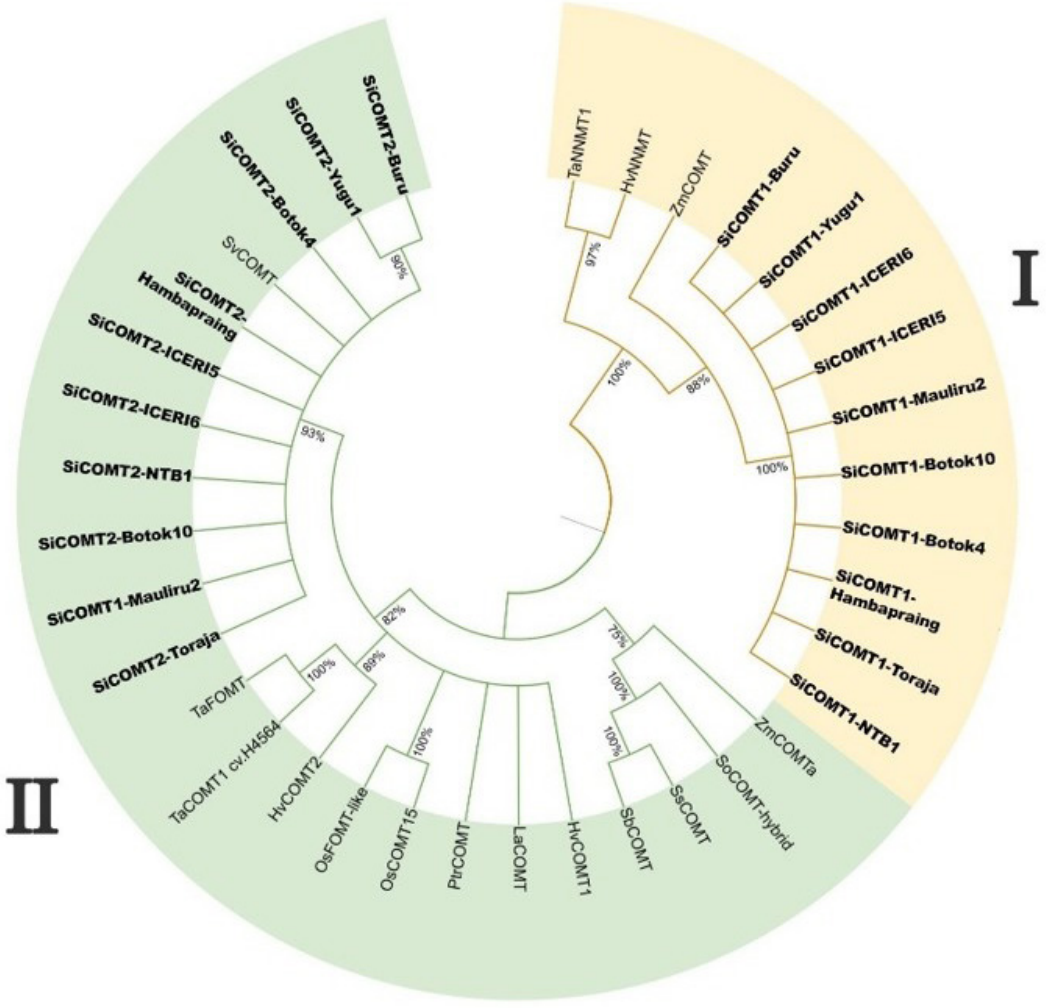

Figure 2. Phylogenetic tree from the *SiCOMT* homologs across species constructed using the maximum likelihood method with 10,000 bootstrap replications

the first and second exons, while synonymous SNPs were found exclusively in the second exon. Protein structure prediction was performed to identify the positions of the non-synonymous variants on the protein structure and to predict the potential effects of the mutation locations (Figure 4).

The mutations included an alanine-to-threonine substitution at position 67 (Ala67Thr) and a proline-

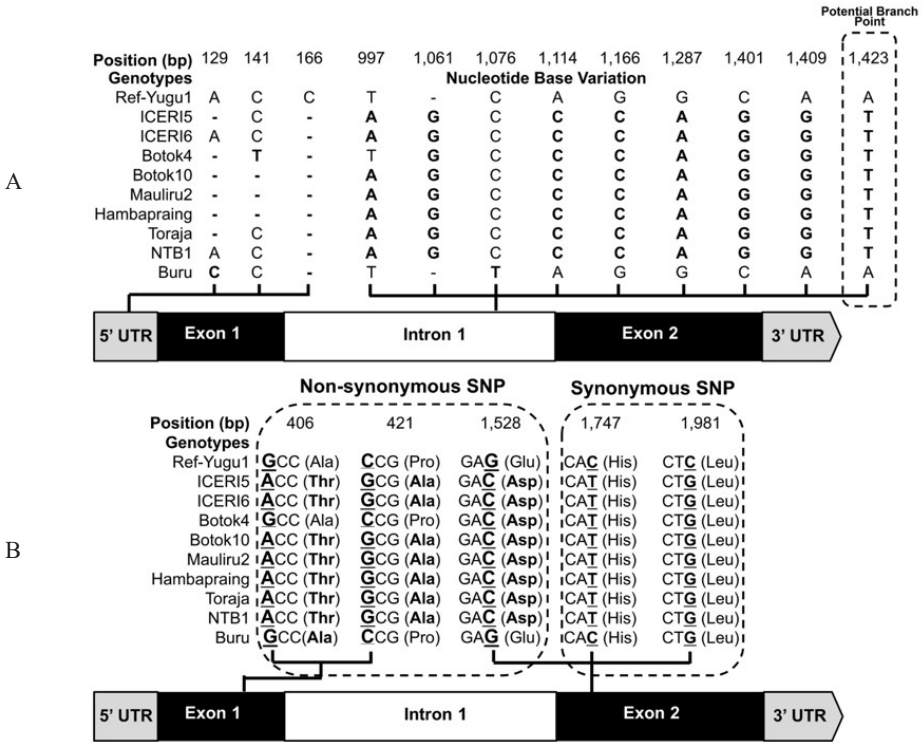

Figure 3. Nucleotide base variation found in the *SiCOMT2* gene from nine Indonesian foxtail millet genotypes in coding regions (A) and non-coding regions (B)

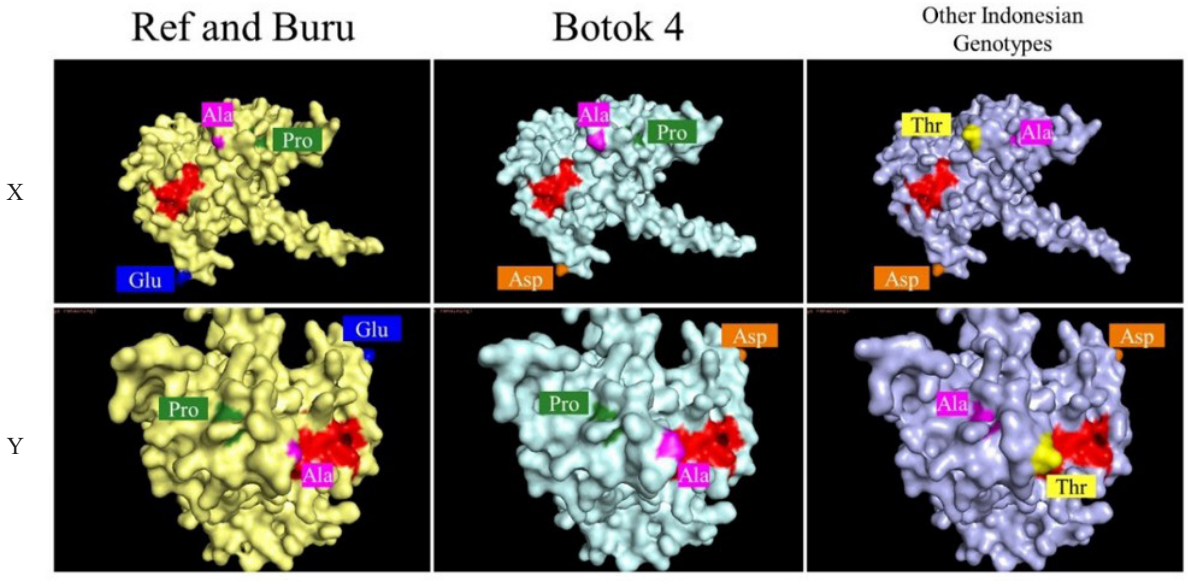

Figure 4. Identification of the mutated amino acid positions in the SiCOMT2 protein. The red region indicates the catalytic site. Individual amino acid residues near the active site are labelled. Panels x and y present different orientations of the superimposed models to illustrate these differences more clearly

to-alanine substitution at position 72 (Pro72Ala), both observed in all Indonesian genotypes except Buru and Botok4. Additionally, a glutamic acid-to-aspartic acid substitution at position 146 (Glu146Asp) was identified in all Indonesian genotypes but was absent in the Buru genotype. No mutation was found in the Buru genotype because its coding sequence was identical to the reference *SiCOMT2* sequence from the 'Yugu1' cultivar. All three mutations are predicted to be located on the protein surface.

Moreover, superimposition between the predicted models against the 'Yugu1' reference structure was performed to evaluate structural differences (Table 2). As 'Yugu1' and Buru share an identical coding sequence, their structural superimposition yielded an RMSD of 0.000 Å. In contrast, the comparison between 'Yugu1' and Botok4 resulted in an RMSD of 0.265 Å, while 'Yugu1' versus the remaining Indonesian genotypes produced a slightly lower RMSD of 0.192 Å. Additionally, superimposition between Botok4 and the other Indonesian genotypes yielded an RMSD of 0.169 Å.

The ΔΔG analysis presented in Table 3 shows that amino acid variations at specific positions may affect protein stability. The Ala67Thr and Glu146Asp mutations indicate neutral effects on protein stability. In contrast, the Pro72Ala mutation is predicted to decrease protein stability.

4. Discussion

Phylogenetic tree analysis revealed that *SiCOMT1* is more closely related to the N-methyltransferase gene group, known to be uninvolved in lignin biosynthesis, than to O-methyltransferase genes from other plant species. This result casts doubt on the classification proposed by Muthamilarasan *et al.* (2015), who had identified *SiCOMT1* as part of the COMT gene based on its sequence similarity to *ZmCOMT* from maize. Lashley *et al.* (2023) explain that N-methyltransferase proteins catalyze the transfer of a methyl group (–CH_3) to a nitrogen atom. However, according to the lignin biosynthesis pathway in the Poaceae family described by Peracchi *et al.* (2024), no nitrogen-containing molecule is directly involved in the process. These findings suggest that *SiCOMT1* may not be involved in lignin biosynthesis, although further functional validation is required. In contrast, the phylogenetic tree showed that *SiCOMT2* is closely related to O-methyltransferase genes, one of the key enzymes in lignin biosynthesis, specifically through the transfer of a methyl group to an oxygen atom (Lashley *et al.* 2023; Peracchi *et al.* 2024). This suggests that *SiCOMT2* may play a more direct role in lignification. Considering that *SiCOMT1* may not contribute to lignin biosynthesis, subsequent discussions focused solely on *SiCOMT2*.

Genes clustered together often exhibit conserved functions and regulatory mechanisms across species (Bharadwaj *et al.* 2021), allowing comparative studies to infer probable roles for *SiCOMT2* based on well-characterized homologs. *SiCOMT2* genes were clustered in cluster II, together with better-studied COMTs from other species, such as barley, sudangrass, wheat, and rice. Knockdown of *HvCOMT1* and *HvCOMT2* via RNAi in barley led to a considerable reduction (~50%) in the S/G lignin ratio (Daly *et al.* 2018). In sudangrass, sequence analysis of the *SsCOMT* gene in the *bmr-12* mutant revealed a nonsense mutation introducing a premature stop codon, resulting in a presumed null allele with significantly reduced gene expression and a marked decrease in acid detergent lignin (ADL) content compared to the wild-type N-12 line (Saballos *et al.* 2008). *SiCOMT2* likely plays an important role in lignin

Table 2. RMSD value of predicted protein structure superimposition

Scenario	Superimposition	RMSD (Å)
1	Yugu1 vs Buru	0.000
2	Yugu1 vs Botok4	0.265
3	Yugu1 vs other Indonesian genotypes	0.192
4	Botok4 vs other Indonesian genotypes	0.169

Other Indonesian genotypes: Botok10, ICERI5, ICERI6, Mauliru2, Hambapraing, NTB1, and Toraja

Table 3. ΔΔG value to predict stability of SiCOMT2 protein

Mutation	Genotypes	ΔΔG (kcal/mol)	Predicted stability change
Ala67Thr	All Indonesian genotypes, except	-0.33	Neutral
Pro72Ala	Botok4 and Buru	-1.15	Decreased
Glu146Asp	All Indonesian genotypes, except Buru	-0.79	Neutral

biosynthesis in foxtail millet, similar to COMT genes in barley and sudangrass, based on its phylogenetic clustering with these better-studied species. Furthermore, a 222-bp InDel in the 3'-UTR of *TaCOMT-3B* defines two allelic variants in wheat, and post-transcriptional regulation linked to this variation was significantly associated with stem lignin content across 157 wheat cultivars (Fu *et al.* 2019). Since *SiCOMT2* is located within the same cluster as *TaCOMT*, it likely shares conserved regulatory elements and structural features with *TaCOMT*. Thus, *SiCOMT2* may be subject to analogous post-transcriptional regulation, potentially influencing lignin accumulation. Moreover, *SiCOMT2* is also closely related to the *OsCOMT15* of rice, which has been reported to exhibit stem-specific expression (Liang *et al.* 2022). Muthamilarasan *et al.* (2015) reported that *SiCOMT2* was highly expressed in leaves, stems, and roots of foxtail millet, implying the key role of COMT in lignification across species.

Although almost identical, the *SiCOMT2* sequences differed slightly among Indonesian foxtail millet genotypes. Nucleotide variations are predominantly located in intronic regions. Zhang (2022) reported that important gene regions (exons or coding regions) mutated less often than other regions (non-coding regions, introns, etc.). Furthermore, Girardini *et al.* (2023) suggested that introns tend to accumulate more mutations due to weaker selective constraints, thereby allowing greater mutational tolerance. Although not translated into an amino acid, an intron functions in regulating gene expression because it contains splice sites and a branch point for splicing. Mutations in that region allow the interruption of the splice site or the branch point recognition in mRNA processing (Cheng *et al.* 2023). A branch point is an adenosine (A) nucleotide base acting as a splicing signal located at a short distance before the 3' splice site (Downs *et al.* 2024). We identified several changes in the adenine (A) base near the 3′ splice site based on the reference sequence, including an A-to-T mutation in the intron at position 1,423 bp. This mutation might act as a branch point, but further investigation at the mRNA level is required to confirm the splicing signals and assess the possibility of alternative splicing. An intronic variant in the *ZmCOMT* gene was significantly associated with stover cell wall digestibility. Given that *SiCOMT2* and *ZmCOMT* were located in the same clade (Figure 2), intronic mutations in *SiCOMT2* may similarly contribute to variation in cell wall composition and digestibility in foxtail millet. While the splicing implications of the intronic mutation remain to be validated in foxtail millet, we also investigated the possible effects of nucleotide variations in the exon on protein structure and stability.

Despite most of the Indonesian genotypes showing three non-synonymous mutations (Ala67Thr, Pro72Ala, and Glu146Asp) when compared to the reference sequence (Yugu1), the superimposition of the predicted models yielded an RMSD of less than 1 Å. An RMSD value below 3 Å from protein superimposition indicates that the protein has high similarity and function (Sapundzhi *et al.* 2022). The low RMSD observed in our models reinforces the likelihood that these mutations do not significantly disrupt the overall protein fold. Moreover, since the mutations are located outside the catalytic site, their impact on protein function is likely limited (Wang *et al.* 2024). Nonetheless, Wang *et al.* (2024) also suggested that even subtle changes in surface topology or pocket flexibility could influence catalytic activity, efficiency, and protein stability.

Furthermore, the Pro72Ala substitution in the SiCOMT2 protein, observed in nearly all Indonesian foxtail millet genotypes, is predicted to decrease protein stability, as indicated by a $\Delta\Delta G$ value below –1.0 (Capriotti *et al*. 2008). A reduction in protein stability increases the susceptibility of the protein to denaturation, functional impairment, and structural abnormalities (Birolo *et al.* 2021). Nevertheless, experimental validation is still required to confirm whether this predicted instability correlates with differences in lignin content or deposition patterns. Based on the superimposition of the predicted protein models (RMSD < 1Å) and the protein stability ($\Delta\Delta G > -1.0$), SiCOMT2 from Botok4 and Buru are predicted to share a similar function and stability with SiCOMT2 from 'Yugu1'. Further comprehensive phenotypic characterization of cell wall composition and digestibility in Indonesian foxtail millet genotypes is essential to elucidate the functional consequences of *SiCOMT2* variation and to validate the predicted molecular effects at the physiological level.

Data Availability

SiCOMT1 (PV271027.1–PV271035.1) and *SiCOMT2* (PV239712.1–PV239713.1; PV271036.1–PV271041.1) gene sequences from nine Indonesian foxtail millet genotypes are available in NCBI GenBank.

Acknowledgements

The author gratefully acknowledges the financial support provided by the Ministry of Higher Education, Science, and Technology of the Republic of Indonesia through Grant No. 006/C3/DT.05.00/PL/2025.

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