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PRODUCTION OF TRANSGENIC *Arabidopsis thaliana* CARRYING THE OIL PALM *Eg4CL1* GENE

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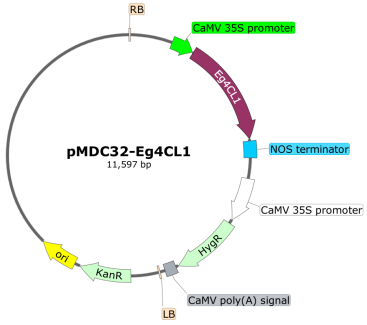
Graphical abstract



Abstract

Oil palm (*Elaeis guineensis*) is a major source of biomass for biofuel production, in addition to yielding edible oil. However, its high lignin content limits biofuel production efficiency, presenting challenges for industrial applications. The oil palm *Eg4CL1* gene has been suggested to play a key role in lignin biosynthesis, but its function remains unverified. Developing transgenic plants that express *Eg4CL1* could aid in understanding its potential role. This study aims to generate transgenic *Arabidopsis thaliana* plants expressing the oil palm *Eg4CL1* gene, which will serve as a tool for functional studies. The 1,623-bp *Eg4CL1* gene was PCR amplified, cloned into the pMDC32 vector, and introduced into Arabidopsis using *Agrobacterium tumefaciens* through the floral dip method. Putative transgenic seedlings were selected on hygromycin-containing MS media and cultivated on substrate for molecular analysis. Ten putative transgenic lines were randomly selected and verified for transgene integration using PCR. Out of 3,000 seeds screened, 70 hygromycin-resistant seedlings were identified. PCR analysis produced a product of target size (≈1786 bp) from all lines analyzed, reflecting stable integration of the *Eg4CL1* gene. These results indicate the genetic transformation of Arabidopsis was successfully accomplished. The establishment of transgenic Arabidopsis expressing *Eg4CL1* will support future functional analysis.

Keywords: Oil palm, *Eg4CL1*, Transgenic, *Arabidopsis thaliana*, Transformation

Abstrak

Kelapa sawit (*Elaeis guineensis*) merupakan sumber utama biomassa untuk pengeluaran biofuel, selain menghasilkan minyak yang boleh dimakan. Walau bagaimanapun, kandungan lignin yang tinggi dalam kelapa sawit menghadkan

keberkesanan pengeluaran biofuel, sekaligus menimbulkan cabaran untuk aplikasi industri. Gen *Eg4CL1* dalam kelapa sawit telah dianggap memainkan peranan penting dalam biosintesis lignin, namun fungsinya masih belum disahkan. Pembangunan tumbuhan transgenik yang mengekspresi *Eg4CL1* boleh membantu dalam memahami peranannya dalam biosintesis lignin. Oleh itu, kajian ini bertujuan untuk menghasilkan tumbuhan transgenik *Arabidopsis thaliana* yang mengekspresi gen *Eg4CL1* dari kelapa sawit sebagai alat untuk kajian fungsional. Gen *Eg4CL1* sepanjang 1,623 bp telah diamplifikasi menggunakan PCR, diklonkan ke dalam vektor pMDC32, dan dimasukkan ke dalam Arabidopsis melalui kaedah transformasi celupan floral menggunakan *Agrobacterium tumefaciens*. Anak benih transgenik yang berpotensi dipilih pada media MS yang mengandungi hygromisin dan kemudian dipindahkan ke substrat pertumbuhan untuk analisis molekul lanjut. Sepuluh andaian transgenik yang dipilih secara rawak dianalisis bagi mengesahkan integrasi transgen menggunakan PCR. Daripada 3,000 biji benih yang disaring, 70 anak benih yang tahan terhadap hygromisin dikenal pasti pada medium selektif. Analisis PCR menunjukkan produk PCR bersaiz sasaran (≈1786 bp) diperoleh daripada semua andaian transgenik yang dianalisis, mencerminkan integrasi stabil gen *Eg4CL1* dalam genom Arabidopsis. Keputusan ini menunjukkan transformasi genetik Arabidopsis berjaya dilaksanakan. Kejayaan pembangunan Arabidopsis transgenik yang mengekspresi gen *Eg4CL1* menyediakan asas kukuh untuk analisis fungsional gen ini pada masa hadapan.

Kata kunci: Kelapa sawit, *Eg4CL1*, Transgenik, *Arabidopsis thaliana*, Transformasi

1.0 INTRODUCTION

Oil palm (*Elaeis guineensis*) is a tropical crop cultivated on a large scale for its oil-rich fruit, which serves as a primary resource in the global production of edible oil [1]. While producing edible oil, the plantations and palm oil processing generate a large quantity of biomass, including empty fruit bunches (EFB) and oil palm fronds [2]. These materials can be utilized as feedstocks for biofuel production [3]. This makes oil palm a crucial contributor to the renewable energy sector, where biofuels derived from oil palm are seen as a more sustainable alternative to fossil fuels. However, oil palm biomass contains lignin, a complex and highly branched polymer found in plant cell walls, particularly in woody tissues. Lignin contributes significantly to structural support, water transport, and resistance to microbial degradation [4-7]. Despite its biological significance, the rigid structure of lignin impedes the efficient conversion of biomass into fermentable sugars, thereby limiting the efficacy of biofuel production [8, 9]. Therefore, while oil palm biomass holds substantial potential for biomass-derived energy, effective strategies to manage lignin content must be developed to improve conversion efficiency.

For biofuel-producing plant species such as sugarcane, poplar and switchgrass, recent research has focused on genetically modifying lignin biosynthetic pathways to optimize biomass utilization [10, 11]. By reducing lignin content in plant tissues, this approach improves biomass extraction and enhances biofuel production efficiency. A fundamental step in this process is the identification of key lignin biosynthetic genes, which serve as targets for genetic manipulation. In recent years, scientists have successfully cloned and characterized several lignin biosynthetic genes in oil palm [12]. Additionally, transcription factors that regulate lignin biosynthesis have been investigated to further refine lignin modification strategies [13]. Other lignin-targeting strategies in different crop species have included downregulation of genes such as *cinnamyl alcohol dehydrogenase* (CAD), caffeic acid *O-methyltransferase* (COMT), and *cinnamoyl-CoA reductase* (CCR), all of which have been shown to alter lignin content and composition [14, 15].

The enzyme 4-Coumarate: CoA ligase (4CL; EC 6.2.1.12) plays a pivotal role within the phenylpropanoid pathway [16], where it catalyzes the activation of hydroxycinnamates by converting them into their respective CoA esters, ultimately leading to lignin biosynthesis [17]. Therefore, regulating the expression of the *4CL* gene provides a potential strategy for modifying lignin content and composition in plants. Genetic modifications targeting *4CL* genes have been explored to enhance plant biomass digestibility for biofuel production by interfering with lignin formation or altering lignin composition. Thus, identifying lignification-related *4CL* gene(s) in oil palm could facilitate lignin modification, improving its suitability for biofuel production. Previously, an oil palm *4CL* gene, termed "*Eg4CL1*" was reported. Yusuf *et al* [18] suggested that *Eg4CL1* plays a crucial role in lignin biosynthesis based on its expression pattern and the *cis*-regulatory elements present in its promoter. However, comprehensive functional studies are still required to confirm its role in lignin biosynthesis.

Transgenic plants expressing the *Eg4CL1* gene serve as essential tools for verifying the specific role of *Eg4CL1* in lignin biosynthesis. By producing these transgenic plants, researchers can assess *Eg4CL1*'s

function in lignin biosynthesis, plant growth, and stress adaptation. Such functional studies provide valuable insights into the potential applications of *Eg4CL1* in engineering plants with optimized lignin content for industrial purposes, including biofuel production. Since the development of efficient floral dip transformation protocols, *Arabidopsis thaliana* has become a popular and widely used model plant for functional gene studies, including those involving species such as oil palm, soybean, and garlic, owing to its well-established genome database, short life cycle, and ease of handling [19-22]. These attributes make it an excellent model plant for studying uncharacterized genetic material. Therefore, Arabidopsis was chosen as the model plant in our study. Accordingly, this study aims to produce transgenic Arabidopsis plants carrying the oil palm *Eg4CL1* gene and providing a platform for future functional analyses and potential applications in plant metabolic engineering. Here, we report the successful production of these transgenic plants using the floral dip transformation method.

2.0 MATERIALS AND METHODS

2.1 Development of Transformation Construct

The *Eg4CL1* gene was PCR amplified from the Eg4CL1 plasmid (obtained from Universiti Putra Malaysia). PCR amplification was performed using the Q5 High-Fidelity DNA Polymerase (New England Biolabs, USA) in a 40 µL total final volume. The reaction mixture contained 1× Q5 reaction buffer, 0.2 mM dNTPs, 0.5 µM of each forward (5'-TAT CTA GAT GGG CCC CTA TCC GCC T-3') and reverse (5'-GCT CTA GAT CAT GGA TGG GGA TCC CC-3') primers, 5 ng of template DNA, 0.4 µL of Q5 DNA Polymerase, and sterile distilled water. PCR was performed using the following thermal cycling conditions: an initial denaturation at 98 ℃ for 30 s, followed by 32 cycles of denaturation at 98 ℃ for 10 s, annealing at 72 ℃ for 25 s, and extension at 72 ℃ for 35 s. Then, a final extension step was carried out at 72 ℃ for 5 min. The PCR product was purified from agarose gel and subsequently digested with the *Xba*I restriction enzyme. The digested DNA fragment was then ligated into the linearized pMDC32 vector using T4 DNA Ligase (Thermo Scientific, USA) to produce the pMDC32-Eg4CL1 construct. The ligation product was transformed into *Escherichia coli* and positive clones were identified by colony PCR and further verified by sequencing. Finally, the transformation construct was introduced into *Agrobacterium tumefaciens* strain GV3101 through the electroporation methods. The electroporation was performed at 2400 V using Electroporator 2510 (Eppendorf, Germany).

2.2 *Agrobacterium* Mediated Transformation of *A. thaliana*

A. thaliana plants were grown to the flowering stage (about eight weeks old) for genetic transformation. *Agrobacterium* cells carrying the pMDC32-Eg4CL1 construct were cultured on LB agar supplemented with 50 µg/mL kanamycin, 30 µg/mL gentamycin, and 25 µg/mL rifampicin. A single colony was inoculated into 5 mL of LB broth containing the previously mentioned antibiotics and incubated overnight at 28 °C, shaking constantly at 250 rpm in the dark. Then, a volume of 0.5 mL from the overnight culture was transferred into a 250-mL flask containing 50 mL of LB broth supplemented with appropriate antibiotics and incubated until the culture reached an OD_{600} of 1.0–1.2. Bacterial cells were then harvested by centrifugation at 3,000 × *g* for 10 min at 4 °C. Following removal of the supernatant, bacterial cells were resuspended in an inoculation medium (5% (w/v) sucrose and 0.02% (v/v) Silwet L-77), and the suspension was adjusted to an OD_{600} of 1.0. The Arabidopsis inflorescences were immersed in the *Agrobacterium* suspension and gently agitated for 10 s. The inoculated plants were covered with a plastic bag to maintain humidity and were kept in the dark for 24 h. The following day, the plastic covering was removed, and the plants were returned to optimum growth conditions (21–23 °C with a 16-h light/8-h dark photoperiod) for one month before seed collection.

2.3 Screening of Putative Transgenic Arabidopsis Carrying the *Eg4CL1* Gene

The harvested Arabidopsis seeds were surface sterilized with 70% (v/v) ethanol and 10% (v/v) sodium hypochlorite for 5 min each, followed by three rinses with sterile distilled water. The seeds were transferred and distributed evenly on Murashige and Skoog (MS) medium (½ strength) supplemented with 20 mg/L hygromycin [23]. The samples were initially stored at 4 °C for 2–3 days. Afterward, they were exposed to light for 8 h at room temperature to promote seed germination before being transferred to dark conditions. After 3–5 days, hygromycin-resistant seedlings with elongated hypocotyls and expanded cotyledons were identified. The seedlings were acclimatized to soil and grown under the optimum conditions as described in Section 2.2.

2.4 Verification of Transgenic Lines

Ten putative transgenic lines were randomly selected for the verification of transgenic status. Young leaf samples were collected from four-week-old putative transgenic plants, and genomic DNA (gDNA) was isolated using a commercially available extraction kit, following the method described by Yusuf *et al.* [12]. PCR was carried out to confirm the presence of the transgene. The reaction was conducted using the DreamTaq Green PCR Master Mix kit (Thermo Scientific, USA) in a total reaction of 20 µL. A positive control reaction was included in parallel. Details of the PCR components and PCR profile are provided in Table 1 and Table 2, respectively.

Table 1 Final concentration and volume of PCR components used to verify the transgenic status of putative transgenic lines

Components	Final Concentration	Volume (µL)
2× Taq Master Mix	1×	10.0
10 µM 35s-Seq47 (5'-TCC TTC GCA AGA CCC TTC-3')	0.2 µM	0.4
10 µM NOSt-Seq (5'-CAA GAC CGG CAA CAG GAT-3')	0.2 µM	0.4
gDNA	30 ng	Variable
Sterile distilled water	-	to 20.0 µL

Table 2 PCR conditions used for the amplification of T-DNA fragment

Step	Temperature (℃)	Time	Cycles
Initial denaturation	95	2 min	1 ×
Denaturation	95	20 s	35 ×
Annealing	57.4	20 s	
Extension	72	55 s	
Final Extension	72	5 min	1 ×

3.0 RESULTS AND DISCUSSION

3.1 Development of Transformation Construct

The 1,623-bp open reading frame [18] of the *Eg4CL1* gene was successfully amplified and cloned into the pMDC32 vector, generating a transformation construct termed "pMDC32-Eg4CL1", which has a total size of 11,597 bp. Sequencing analysis confirmed that no mismatches were present in the insert. The pMDC32-Eg4CL1 construct was designed for heterologous expression of the *Eg4CL1* gene in *A. thaliana*. The *Eg4CL1* gene was inserted into the pMDC32 vector, flanked by the CaMV 35S promoter and the NOS terminator. This will allow ubiquitous expression of the *Eg4CL1* gene in a transgenic plant as the CaMV 35S promoter is known for its constitutive promoter [24]. In addition, the pMDC32-Eg4CL1 construct harbors a hygromycin resistance cassette, serving as a marker for identifying transgenic lines. The plasmid map of the pMDC32-Eg4CL1 construct is illustrated in Figure 1. Previously, the pMDC32 vector has been used in the study by Hilman *et al.* [25] to generate transgenic tobacco plants that overexpressed the *EgCAD2* gene derived from oil palm.

3.2 *Agrobacterium* Mediated Transformation of *A. thaliana*

In the present study, wild-type Arabidopsis plants were cultivated under optimum conditions for genetic transformation, as described in the Materials and Methods section. Figure 2(a) shows that the plants developed healthy rosette leaves five weeks after being cultivated on soil. Under our cultivation conditions, the Arabidopsis plants entered the reproductive phase at approximately eight weeks of age. At this stage, inflorescence stems developed from the plants, as shown in Figure 2(b). Figure 2(c) shows that floral buds emerged from the shoot apical meristem and developed into flowers. Floral dip transformation was performed on the Arabidopsis plants at this stage. After transformation, siliques developed from the spent flowers on the inflorescence stems, as shown in Figure 2(d), and the transformed plants were maintained until seed maturation. Figure 2(e) shows a transformed plant contained within an Aracon. This setup prevents cross-pollination between adjacent plants and seed loss, thereby allowing efficient seed collection.

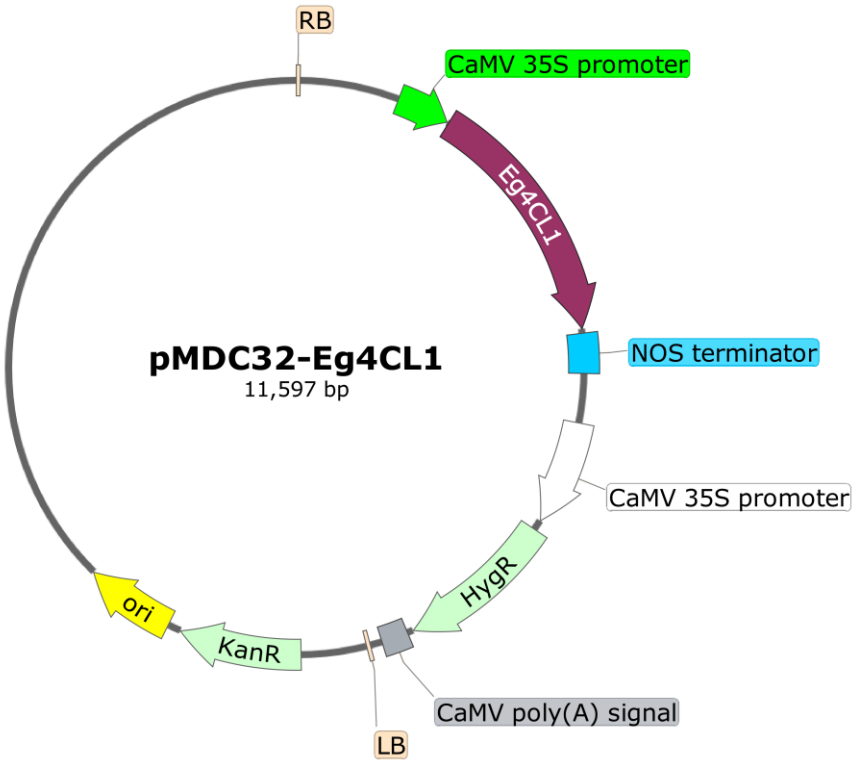


Figure 1 Plasmid map of the pMDC32-Eg4CL1 construct used for genetic transformation

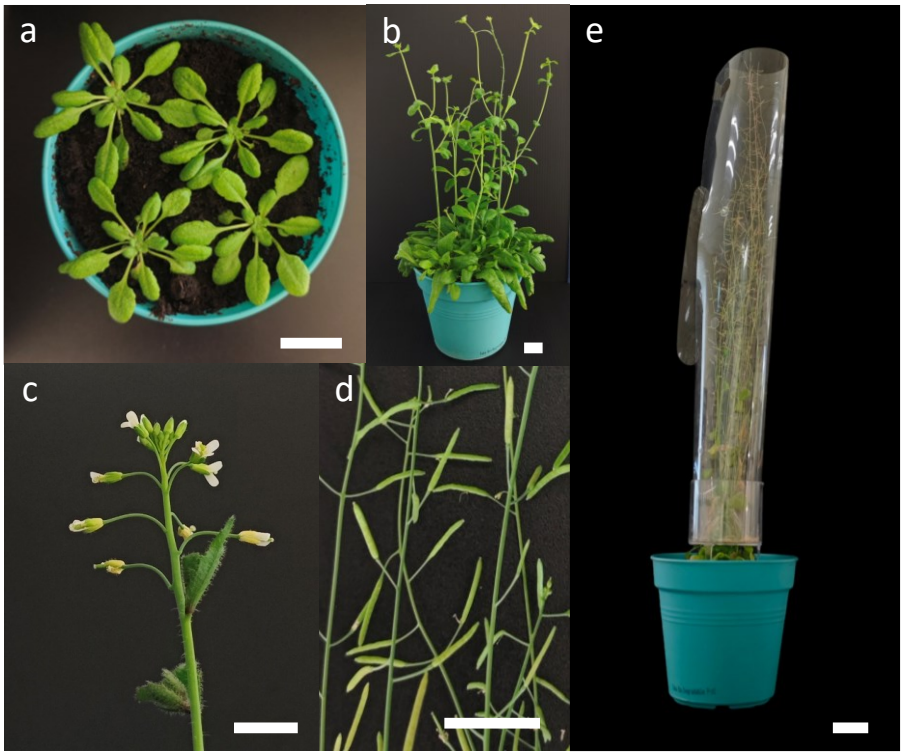


Figure 2 Developmental stages of *A. thaliana* plants used in genetic transformation. (**a**) Five-week-old plants with healthy rosette leaves. (**b**) Inflorescence stems elongated as the plants transitioned to the reproductive stage. (**c**) Floral buds emerged and progressed into fully developed flowers. (**d**) Siliques formed following floral dip transformation. (**e**) A transformed plant enclosed in an Aracon to prevent unintended pollination and seed dispersal. Scale bar = 2 cm

3.3 Screening of Putative Transgenic Arabidopsis Carrying the *Eg4CL1* Gene

Approximately 3,000 seeds derived from *A. thaliana* plants (T_0) that underwent transformation were cultured on half-strength MS medium supplemented with 20 mg/L hygromycin. Phenotypic differences became apparent in the Arabidopsis seedlings approximately five days after germination. Based on their phenotypes, the seedlings could be classified into two groups: hygromycin-resistant and hygromycin-sensitive. The former thrived on the selective medium, exhibiting elongated hypocotyls and roots, along with expanded cotyledons. In contrast, the latter displayed stunted growth. Hygromycin is an aminoglycoside antibiotic that impairs ribosomal translocations, leading to ribosomal misreading. Thus, it inhibits protein synthesis and eventually causes cell death [26, 27]. The hygromycin resistance gene encodes hygromycin B phosphotransferase (HPT), which phosphorylates hygromycin, rendering it inactive and preventing its toxic effects [28]. As a result, transgenic seedlings carrying the hygromycin resistance gene survive in the presence of hygromycin, while non-transgenic seedlings exhibit severe growth inhibition. Figure 3(a) shows the putative transgenic (hygromycin-resistant) and non-transgenic (hygromycin-sensitive) Arabidopsis seedlings cultivated on the selective medium. A total of 70 hygromycin-resistant seedlings (T_1) were obtained from the 3,000 seeds screened, yielding a transformation efficiency of 2.3%. This efficiency is within the range reported for the *Agrobacterium* floral-dip method. It is worth noting that transformation efficiency depends on several factors, such as bacterial strain, inoculum density, surfactant, and plant ecotype [29-31]. Several studies have reported high transformation efficiencies, whereas some protocols report lower efficiencies (~0.1–0.2%) [32]. The putative transgenic seedlings were transferred to growing substrate, as shown in Figure 3(b), and cultivated for further molecular analysis. Molecular analysis is required to verify the stable integration of the *Eg4CL1* gene in the Arabidopsis genome.

3.4 Verification of Transgenic Lines

As a large number of putative transgenic plants were obtained, only 10 lines were randomly selected for PCR verification. The primers used in the PCR analysis were designed to anneal to the CaMV 35S promoter and the NOS terminator, generating an expected 1,786-bp PCR product. The use of primers targeting the CaMV 35S promoter and NOS terminator ensures that the amplified PCR products derive from the transgene rather than endogenous *At4CL* genes, as the *Eg4CL1* gene might share sequence homology with the *At4CL* genes. Figure 4 shows the PCR products amplified from the gDNA of the selected transgenic lines. As anticipated, a PCR product of the expected size (≈1786 bp) was detected in all analyzed transgenic lines (lanes 1-10). The bands were identical in size to the positive control (lane 11). The results confirm the presence of the *Eg4CL1* gene in these transgenic lines. In other words, the oil palm *Eg4CL1* gene has stably integrated into the Arabidopsis genome.

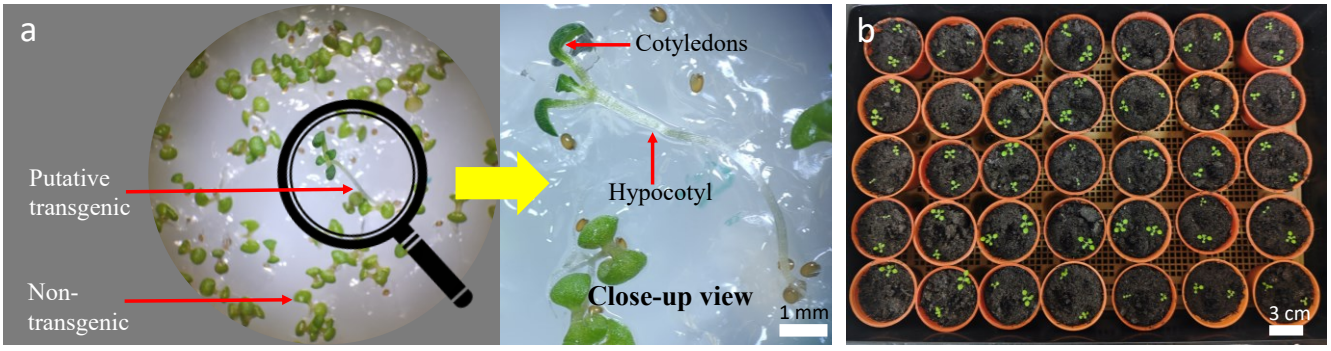


Figure 3 Selection of putative transgenic and acclimatized *A. thaliana* seedlings. (**a**) Putative transgenic seedlings thriving on a selective medium containing 20 mg/L hygromycin. Close-up view of a putative transgenic seedling. (**b**) Putative transgenic seedlings cultivated in growing substrate

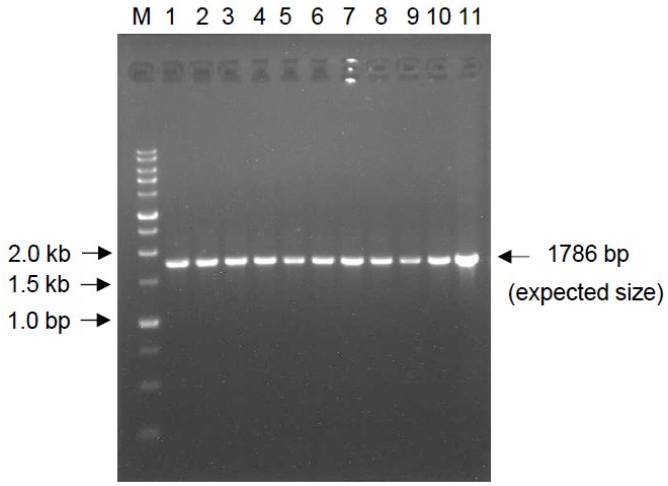


Figure 4 PCR verification of selected transgenic lines. Lanes 1-10: transgenic lines; Lane 11: positive control. M: 1 kb DNA ladder (1st BASE, Singapore), used as a size reference for the DNA fragments.

Although transgenic Arabidopsis lines carrying the *Eg4CL1* gene have been successfully generated in the present study, further analyses are required before these transgenic plants can be used for functional studies. This is because PCR verification only confirms the presence of the transgene but does not indicate its copy number or expression level. Segregation analysis is needed to examine the inheritance of the transgene and to identify homozygous transgenic lines for subsequent experiments. In addition, gene expression analysis is required to confirm that the gene is actively transcribed and translated in the plant.

Once the transgenic lines have been molecularly validated, functional studies can be conducted to determine the effects of *Eg4CL1* overexpression. Analyses such as lignin quantification, histological examination, and phenotypic evaluation will be important for validating the proposed role of *Eg4CL1* in lignin biosynthesis. Overexpression of *Eg4CL1* may enhance lignin deposition, potentially affecting cell wall rigidity, mechanical strength, and responses to environmental stresses

Previous functional studies on *4CL* genes from various plant species have validated their involvement in lignin biosynthesis [33, 34]. Therefore, manipulation of this particular gene may alter lignin content and composition, with potential implications for biomass quality, biofuel production, wood properties, and plant resistance to pathogens and environmental stresses.

Future research will focus on phenotypic evaluation and metabolic profiling to determine the effects of *Eg4CL1* overexpression. These studies, including lignin quantification, histological analysis, and phenotypic evaluations, will be addressed in future work to better elucidate the role of this gene in lignin biosynthesis.

4.0 CONCLUSION

In conclusion, transgenic Arabidopsis plants carrying the oil palm *Eg4CL1* gene were successfully generated in this study. The *Agrobacterium*-mediated floral dip transformation method proved to be an effective approach for introducing foreign DNA into *A. thaliana*. The successful incorporation of the *Eg4CL1* gene into the Arabidopsis genome was confirmed by PCR analysis. While functional role of the gene was not assessed here, this work provides the foundation for future studies on the functional characterization of *Eg4CL1* and its potential role in the phenylpropanoid pathway, particularly in lignin biosynthesis. It could have applications in improving oil palm traits or other crop species through genetic engineering.

Ethics Statement

This research did not involve human participants or animal subjects. All procedures involving transgenic *A. thaliana* were conducted in compliance with the regulations of the Genetic Modification Advisory Committee (GMAC), and all plant materials were handled under appropriate biosafety containment.

Authors Contribution

Chong Yu Lok Yusuf conceived and designed the study. Omar Nawawi and Mohamad Shafek Hilman conducted the experiments and collected the data. Dehong Xie, Cuixian Zhang, Huaifeng Yi and Mohd Norsazwan Ghazali provided critical insights into methodology and data interpretation. Omar Nawawi drafted the manuscript, while Chong Yu Lok Yusuf, Noor Azmi Shaharuddin, and Mat Yunus Abdul Masani critically reviewed and finalized the manuscript. All authors approved the final version of the manuscript.

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Conflict of interest

The authors state that they have no competing interests.

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