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Cover Page Footnote

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Transformation of Inhibitor of Meristem Activity (IMA) Gene into *Jatropha curcas* L.

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Abstract

Jatropha is one of the many biodiesel plants developed in tropical countries. Efforts to increase its productivity can be done using various methods of breeding. One of the breeding methods is the introduction of genes into the *Jatropha* plant. The aim of this study is to assess the success of genetic transformation using the Inhibitor of Meristem Activity (*IMA*) gene in *Jatropha curcas*. The research procedures included inoculation of explants with *Agrobacterium tumefaciens*, callus induction, screening test of selection media, regeneration, and gene expression analysis using Polymerase Chain Reaction (PCR). *IMA* is one of the genes that controls flowering genes and ovule development. It was first isolated from tomato plants and has been successfully overexpressed in these plants using the *Cauliflower Mosaic Virus* (CaMV) 35S promoter. In this experiment, plant transformation was performed on *J. curcas* as the target. Explant callus formation in both the control and treated samples was good, but shoot formation decreased dramatically in the treated explants. PCR analysis indicated that *IMA* genes can be inserted into *J. curcas* with the size of the *IMA* gene is 500 bp.

Abstrak

Transformasi Gen Inhibitor of Meristem Activity ke Tanaman *Jatropha curcas* L. Jarak Pagar merupakan salah satu tanaman penghasil biodiesel yang banyak dikembangkan di beberapa negara tropis. Usaha peningkatan produktivitasnya terus ditingkatkan dengan berbagai metode pemuliaan. Salah satu metode pemuliaan tersebut adalah dengan menyisipkan gen-gen yang unggul ke tanaman Jarak Pagar. Penelitian ini bertujuan untuk mengetahui tingkat keberhasilan transformasi genetik yang menggunakan gen *IMA* (*Inhibitory Meristem Activity*) ke dalam tanaman *Jatropha curcas* L. Tahapan penelitian meliputi inokulasi eksplan dengan *Agrobacterium tumefaciens*, induksi kalus, seleksi, regenerasi, dan analisis ekspresi gen *IMA* dengan metode *Polymerase Chain Reaction* (PCR). Gen *IMA* merupakan salah satu gen yang dapat mengontrol pembungaan dan perkembangan ovul. Gen ini diisolasi pertama kali dari tanaman tomat dan peningkatan ekspresi telah berhasil dilakukan pada tanaman tomat itu sendiri menggunakan promoter 35S *Cauliflower Mosaic Virus* (CaMV). Pada percobaan ini dilakukan transformasi gen ke dalam tanaman target *J. curcas*. Pembentukan kalus pada menunjukkan hasil yang baik eksplan kontrol dan eksplan yang mengalami perlakuan, tetapi pada eksplan perlakuan hasil sangat menurun pada tahap pembentukan tunas. Analisis dengan PCR mengindikasikan bahwa gen *IMA* dapat disisipkan ke *J. curcas* dengan ukuran gen *IMA* sebesar 500 bp.

Keywords: *biodiesel, inhibitory, Jatropha, meristem*

Introduction

Fuel prices are unstable and the supply of fossil-derived fuels is diminishing, leading to increased efforts to find alternative energy sources. An example of an alternative energy source derived from plants is the physic nut plant (*J. curcas* L.). This plant has many advantages as a source for the production of alternative energy: It can grow in a variety of soil types, does not require a lot of

water, produces non-edible seeds that contain 30-40% oil (ideal for biodiesel production) and can also be used to produce pesticides [1].

J. curcas is grown in South America, Central America, Asia and Africa [2]. The choice of *J. curcas* as a primary source of biodiesel is appropriate because it is not consumed as food [3], is able to grow well in dry environments [4], [5] and has ecological benefits

associated with its ability to store water [6]. *Jatropha* can also be planted with woody plants using the intercropping system [7].

Increased production and oil content are the main goals in breeding *J. curcas* [1]. Genetic engineering can be used to improve the productivity of *J. curcas* plants [8]. The oil content of seeds can be improved with the introduction of diacylglycerol acyltransferase genes [9], while efforts to increase the number of seeds have involved the introduction of heading date 3a (*Hd3a*) flowering genes [10] such as the PACS (*Pseudomonas aeruginosa* Citrate Synthase) gene. And to resist aluminum toxicity [11], the aluminum-activated malate transporter (*ALMT*) gene is used [12]. Now, *Jatropha* genetic improvement efforts are focusing on the use of the *IMA* gene to understand the plant's meristem activity. Genetic engineering in plants relies on regeneration from calluses. To achieve regeneration of transgenic plants, careful attention is paid to the selection of media, growth regulators and explants. In the case of the *Oryza japonica* rice plant, which is used as a model plant for transformation, the scutellum is used as an explant [13]. Young cotyledons are preferred for genetic transformation in *J. curcas* because they are more susceptible to *Agrobacterium* infection [14]. In vitro plant regeneration of *J. curcas* has been achieved by several researchers using various explants including hypocotyl [15] and cotyledons [14,16].

The *IMA* gene, isolated from the tomato plant, regulates flowering and ovule development. The gene acts as a repressor of genetic expression. In the control group, WUSCHEL (WUS) meristem center is determined by the nucellus during ovule development. The *IMA* gene inhibits cell proliferation during floral termination, controls the number of carpels produced during flower development and plays a role in the initiation of the primordial ovule, which is activated by type-D gene expression. In addition, *IMA* is involved in several hormone signaling pathways, as an important effector in controlling the trajectory of meristem activity associated with cleavage of differentiation and control growth hormone [17]. This study aims to evaluate the success of genetic transformation in *J. curcas* using the *IMA* gene.

Materials and Methods

Explants and inoculum preparation: The plant material used in this study is physic nut (*J. curcas* L.) from Pakuwon, Sukabumi, West Java Province, Indonesia. The seeds were shelled and sterilized using 0.5% (v/v) sodium hypochlorite for 15 minutes, washed several times with distilled water and dried on sterile tissue. Seeds were grown on medium, concentration of ½MS [18] supplemented with 20 g/L sucrose and 8 g/L of pure agar for 8 days. Single colonies of *A. tumefaciens* LBA4404 carrying gene constructs of *IMA* genes were

grown on medium containing 10 µg/mL spectinomycin [17]. Bacterial cultures were shaken with a shaker at 200 rpm at a temperature of 28 °C for 48 hours.

Introduction of *IMA* genes to *J. curcas* with *A. tumefaciens*: Transformation was done using cotyledons as explants with some modifications [16]. *Jatropha* transformation involves several stages: preparation of cotyledons; infection; co-cultivation; callus induction; shoot induction (regeneration); elongation of shoot; and rooting. For explant preparation, sterile seeds were planted on ½MS medium [18] and grown for 12 days. *A. tumefaciens* cultured in liquid Luria Bertani (LB) medium containing 10 µg/mL of spectinomycin was incubated at room temperature for 48 hours with shaker. Bacterial cells were separated from the liquid medium using a centrifuge at 5,000 rpm for 5 minutes. The pellet was then dissolved in MS medium containing 20 mg/L acetosyringone and re-suspended at OD₆₀₀ = 0.5. 1 cm² cotyledons was infected by soaking on *A. tumefaciens* bacterial suspension for 10 minutes with shaker. The cotyledons were then dried with sterile tissue and grown on a solid MS co-cultivation medium containing 20 mg/L acetosyringone. Cultures were maintained in a dark room for 3 days. The cotyledons were then washed in sterile water and soaked in a solution of cefotaxime 500 mg/L to kill the *Agrobacterium* bacteria. For callus induction, cotyledons were grown on callus inducing medium (CIM) containing 0.2 mg/L myo-inositol, 10 mg/L thiamine, 0.05 mg/L BAP, 0.1 mg/L IBA and PVP 5 g/L with pH 5.8 medium and incubated in dark conditions at 25 °C for 21 days. The total number of calluses emerging from the explants was then calculated. Regeneration was done by moving the callus cotyledon on CIM and placing it in lighted conditions of 2,000 lux. At this stage, 200 mg/L cefotaxime and 20 mg/L kanamycin were added to the CIM. The number of shoots that appeared was calculated in the first and second months of treatment. Shoots that grew on selection media were then transferred into ½MS medium, 2 mg/L IBA, 100 mL/L 0.1M AgNO₃: Na thiosulfate (1:4) and subculture every 10 days. For root formation, shoots were grown on the same medium without growth hormone [14]. Plantlets were acclimatized in rice husk and covered with plastic for 1 week.

The number of callus explants and the number of shoots grown from the callus were used to determine the efficiency of transformation. To calculate the percentage of callus explants, the number of calluses was divided by the number of cultured explants. Transformation efficiency was calculated from the number of explants that produced shoots in selection medium divided by the number of explant calluses.

***J. curcas* leaf DNA extraction.** Putative transgenic plants that are selected as transformed (using an antibiotic as selection agent) were then analyzed by

PCR. DNA was isolated by taking 0.1 g of leaf and grinding it until smooth in a mortar, adding 650 mL of extraction buffer (2% CTAB, 0.1 M Tris HCl pH 9.5, 20 mM EDTA, 1.4 M NaCl and 2% PVP) and 5 mL β -mercaptoethanol and placing them into 1.5 mL tubes. Extracts were incubated at 65 °C for 30 minutes, inverted every 10 minutes. The DNA extract was placed on ice for 5 minutes and 1 times the volume of chloroform to isoamylalcohol (24:1) was added and inverted 8 times. The extract was then centrifuged at a speed of 10,000 rpm for 15 minutes at 4 °C. The supernatant was transferred to a new tube and added to an amount equivalent in volume to phenol: chloroform: isoamylalcohol, (25:24:1). After being centrifuged at 10,000 rpm for 15 minutes, at 4 °C, the supernatant was transferred into a new tube and added to 0.1 times the volume of 2 M Na acetate with pH 5.2, and 2 times the volume of absolute ethanol for DNA precipitation. The solution was stored at -20 °C overnight. DNA was precipitated by means of centrifugation at 10,000 rpm for 30 minutes at 4 °C. The DNA precipitate was rinsed in 500 mL of 70% ethanol, and centrifuged at the same speed for 5 minutes. After drying, the DNA was dissolved again by adding 20 mL ddH₂O. To keep the DNA from being contaminated by RNA, 5 mL RNase (10 mg/mL) was added to degrade the RNA and incubated at 37 °C for 10 minutes, then inactivated at 70 °C for 10 minutes.

Detection of transgenic plants: The *IMA* gene was analyzed using 35S CaMV for forward primer and *IMA* for reverse primer. PCR conditions included the following: pre-PCR 95 °C for 4 minutes; denaturation 94 °C for 30 seconds; annealing at a temperature of 55 °C for 45 seconds; and elongation at 72 °C for 5 minutes. This cycle was repeated 35 times with a post-PCR temperature of 72 °C for 5 minutes and temperature of 20 °C for 5 minutes. *IMA* gene detection was made using primers CaMV-35S 5'AGGGATGACGCACAATCC3' (forward) and 5'TCATTTAGTAGTAGAAGAAG3' (reverse).

Results and Discussion

Callus formation started at day 14, and not all of the calluses produced shoots. Some calluses from explants produced multiple shoots. Kanamycin was not used in callus production because *J. curcas* is very sensitive to kanamycin. Previous research has shown that a concentration of 5 mg/L of kanamycin in medium can inhibit the process of callus formation [14].

Cefotaxime was used in growing the CIM in order to reduce the population of *Agrobacterium*. If the population of *Agrobacterium* in the medium is too high, it can cause the calluses to die. Therefore, one should consider carefully the type of *Agrobacterium* to be used

during the construction of the expression vector. In the control sample, 40 explants produced 35 calluses; the 100 treated explants produced 70 calluses. A common problem in the maintenance of calluses is overgrowth of *Agrobacterium*, which harms the explant's ability to develop into a callus.

Agrobacterium infection of cotyledons can occur within 20 minutes. Infection will be successful if there is a wound on the explants (Figure 1a). Co-cultivation was conducted for 3 days using the bacterial concentration OD₆₀₀ = 0.1 in different concentrations, based on previous research [14,16]. In the first 4 weeks of using CIM (with 300 mg/L cefotaxime), co-cultivation with *Agrobacterium* was used to induce the callus (Figure 1b). Regeneration of *J. curcas* callus was done using MS medium, BA 3 mg/L and IBA 0.01 mg/L [19]. The physiological condition of the explant source determines the ability of the explants to produce callus. The genotype and explant types show different results in callus production using the same medium. Explants in CIM can produce shoots, but they must be kept in lighted conditions [16]. Furthermore, it was reported that the addition of 1 mg/L 2, 4-dichlorophenoxy-acetic acid (2, 4-D) or 1 mg/L NAA in the medium can induce callus from cotyledons of *Jatropha*, but the resulting callus cannot regenerate to form buds. Calluses capable of regenerating can be obtained from the medium with the addition of 0.05 mg/L IBA combined with 1.5 mg/L BA.

Shoot-inducing medium (SIM) with 20 mg/L kanamycin and 100 mg/L cefotaxime was used to select transformants. Kanamycin-resistant shoots were obtained after 6-8 weeks in the SIM selective medium (Figure 1c). In this study, we used only the first transformant and selection was done using kanamycin with a concentration of 20 mg/L to obtain a total of 65 shoots from the control and 13 shoots from treated *J. curcas* (Figure 2).

The main difficulty in the tissue culture of *Jatropha* is in the rooting stage. In this stage a rooting medium (RM) of ½ MS with 2 mg/L IBA, 20 mg/L kanamycin and 100 mg/L cefotaxime was used for 4-5 weeks [14]. The study managed to obtain rooted plantlets (Figure 1d, 1e) and acclimatization (Figure 1f). A total of 87.5% of the control explants of nut accessioned IP-2P callus, while 70% of treated explants grew successfully (Figure 2).

In the *Jatropha*'s rooting stage, the plants were grown in ½MS medium without growth regulators. Five of 9 buds (55.5%) were able to produce roots. Modifications to rooting were done by adding 20 mL of BAP, AgNO₃ and vitamin C; this was sufficient to induce rooting. Sophisticated research methods typically get 1 plantlet from 120 shoots in vitro [14]. Using bispyribac in selection media can improve the efficiency of the genetic

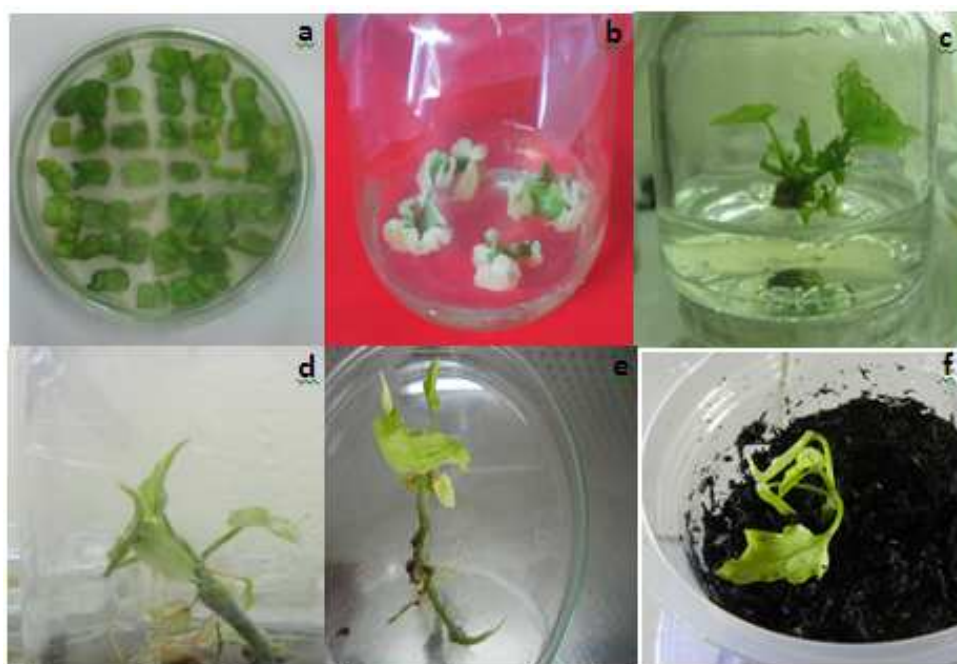


Figure 1. IMA Genes are Introduced into *J. curcas* Plants in Six Stages: (a) co-cultivation of Explants with *A. tumefaciens* for 3 Days; (b) Induction of Callus using callus Induction Media; (c) and (d) Plantlets in rooting Media; (e) Plantlets ready for Acclimatization; (f) during Acclimatization

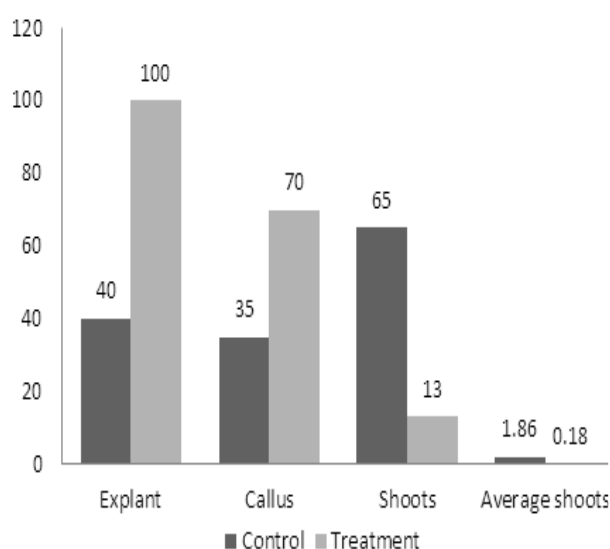


Figure 2. Comparison of the Development of Explants into Shoots

transformation [20]. Some 9.6% of shoots formed tolerance to kanamycin 20 mg/mL. Previous studies have reported rates as high as 15-20%. The difference could be due to the use of different *Jatropha* accessions.

Genetic transformation of *Jatropha* using *A. tumefaciens* was successfully performed using cotyledons as the explant source [16]. In this study, cotyledon explants

were selected because of their callus-producing abilities. In addition, cotyledons are more responsive to infection by *A. tumefaciens* than other parts of the embryo such as the petiole, hypocotyl and epicotyl. They are also easily obtainable at short notice [21].

Analysis of Transgenic Plants: Putative transgenic plants can be confirmed using the PCR method. The primers used to confirm the presence of transgenic plants were CaMV 35S for forward primers and the *IMA* gene for reverse primers. Plant DNA was isolated from 40 samples; 20 samples of the plants obtained had good quality of DNA. PCR using 35S CaMV and *IMA* produced 500 bp bands, while PCR using gene-specific forward and reverse primers produced an *IMA* of 272 bp (Figure 3).

The PCR results showed the size of the band at 500 bp using CaMV-35S primers and *IMA* reverse on lines 5,6,7,9 and 14. On line 4, gene-specific primers containing the *IMA* gene were measured at 272 bp. Increasing the number of shoots can increase the chances of successfully producing transgenic plants. The addition of growth regulators in the culture medium (thidiazuron) increased the number of shoots in vitro *J. curcas* to 17 [22].

In this study, we have demonstrated that *IMA* genes can be inserted into the genome of *J. curcas* based on PCR analysis. We plan to do a qPCR analysis to verify the number of gene copies that will be amplified.

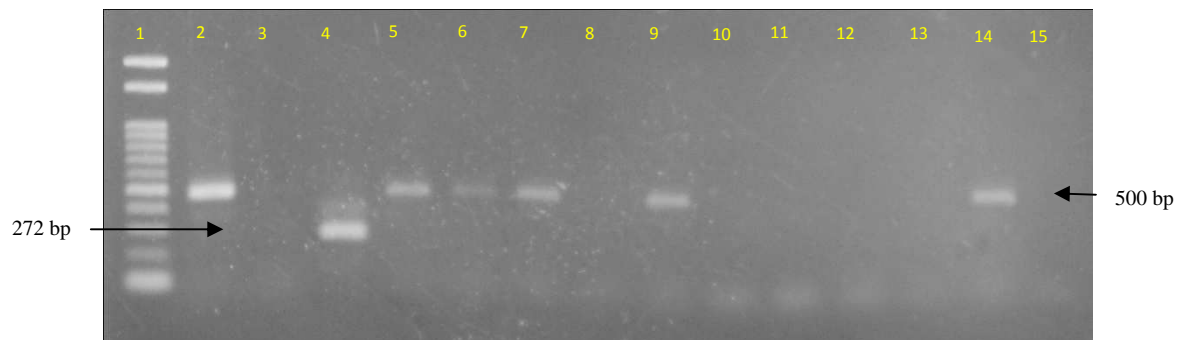


Figure 2. (1) Marker 100 bp; (2) Plasmid using 35S and IMA Reverse Primer; (3) Wild Type; (4) Plants using Gene-Specific Primers; (5), (6), (7), (9) and (14) Putative Transgenic Plants using the Primers 35S CaMV and IMA Reverse; and (10), (11), (12), (13), (15) and (16) Non-Transgenic Plants

Conclusion

IMA genes can be inserted into *J. curcas*, based on a PCR analysis that showed the size of the *IMA* gene in the *J. curcas* genome is 500 bp.

Acknowledgments

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