Transformation of *Jatropha curcas* L. for production of larger seeds and increased amount of biodiesel

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Abstract  The development of green energy is important to mitigate global warming. *Jatropha (Jatropha curcas L.)* is a promising candidate for the production of alternative biofuel, which could reduce the burden on the Earth’s resources. *Jatropha* seeds contain a large quantity of lipids that can be used to produce biofuel, and the rest of the plant has many other uses. Currently, techniques for plant genetic transformation are extensively employed to study, create, and improve the specific characteristics of the target plant. Successful transformation involves the alteration of plants and their genetic materials. The aim of this study was to generate *Jatropha* plants that can support biofuel production by increasing their seed size using genes found via the rice FOX-hunting system. The present study improved previous protocols, enabling the production of transgenic *Jatropha* in two steps: the first step involved using auxins and dark incubation to promote root formation in excised shoots and the second step involved delaying the timing of antibiotic selection in the cultivation medium. Transgenic plants were subjected to PCR analysis; the transferred gene expression was confirmed via RT-PCR and the ploidy level was investigated. The results suggest that the genes associated with larger seed size in *Arabidopsis thaliana*, which were found using the rice FOX-hunting system, produce larger seeds in *Jatropha*.

Key words: biodiesel, cDNA overexpression, *Jatropha curcas* L., rice FOX-hunting.

Introduction

Because the energy produced through biofuel is both abundant and flexible, research into biofuel production has rapidly increased. Many organisms are capable of naturally producing biofuel; for example, thermophilic bacteria (Lin and Xu 2013), cyanobacteria, and microalgae (Parmar et al. 2011) as well as land plants such as sugarcane (*Saccharum officinarum*) and maize (*Zea mays*) have been used to produce bioethanol for the purposes of fuel (Byrt et al. 2011).

*Jatropha* (*Jatropha curcas L.*) or physic nut is a perennial shrub native to Mexico and Central America; it can also be cultivated in arid or semi-arid environments in Africa, Asia, and South America (Montes Osorio et al. 2014). *Jatropha* plants can be used for many purposes: their latex can be used for wound healing and to treat some skin diseases, their leaves can be used for silkworm feeding and as anti-inflammatory medicine, their fruit and hull can be used for biogas production and as combustibles, their seed cake can be used as fertilizer and animal feed, and their seed oil can be used for soap production, as insecticides and molluscicides, and importantly, as a resource for biofuel production because *Jatropha* seeds can serve as partial or full replacements of petroleum-based diesel fuel (Forson et al. 2004; Gübitz et al. 1999). Nonetheless, *Jatropha* has a long-life cycle; the period from its sprouting to the yield of flowers and fruits is long (Achten et al. 2010). However, its seed yield is not stable and is dependent on the growing conditions.
and environment, due to which it cannot meet human expectations.

Many studies have attempted to address these obstacles, particularly studies of Jatropha flowers, fruits, and seeds, such as those focusing on the promotion of flower production by increasing the concentrations of endogenous cytokinins involved in flower development (Ming et al. 2020) and ovule development-related gene expression (Xu et al. 2019). Seed quality (i.e., size, oil content, and oil component) is important with regards to biofuel production (Ruan et al. 2012). The ability to increase Jatropha seed size is a promising sign for biofuel production. The manipulation of auxin response factor 19 (ARF19) in Jatropha has been shown to lead to larger seeds (Sun et al. 2017). Additionally, the ability to alter the lipid content of Jatropha seeds by increasing the expression of transcription factors related to specific lipid synthesis (Ye et al. 2018) and lipid composition at each stage of maturation has also been studied (Jonas et al. 2020; Sinha et al. 2015).

Previously, the expression and function of genes have been studied in Arabidopsis thaliana using the loss-of-function method; however, some genes play a critical role in plant survival and the loss-of-function of these genes is lethal. Alternatively, the gain-of-function method has been used to support the study of mutants that cannot be isolated via the loss-of-function method (Nakazawa et al. 2003). This has led to the development of a novel gain-of-function system known as the full-length cDNA overexpressing gene hunting (FOX-hunting) system (Ichikawa et al. 2006). Sakurai et al. established the rice FOX-hunting system by introducing rice full-length cDNAs into Arabidopsis plants via Agrobacterium tumefaciens infection of cotyledon explants using the herbicide phosphinothricin as the selection agent (Li et al. 2008a).

LOC_Os08g43210 is the gene that encodes probable inositol transporter 2. In Arabidopsis, the protein encoded by this gene is localized at the plasma membrane and mediates the symport of H+ and several inositol compounds, such as myoinositol, scyllo-inositol, D-chiro-inositol, and muco-inositol (Schneider et al. 2007). The inositol phospholipids play an important role in signaling and transportation between the plasma membrane and the endoplasmic reticulum, which regulate cell growth and proliferation (Stevenson et al. 2000). Arabidopsis seeds contain inositol, such as phosphatidyl inositol, phytoglycolipids, and ceramide-phosphate-polysaccharide (Carter and Kusic 1969).

LOC_Os08g41910 is the gene that encodes the Sua5/YciO/YrdC/YwlC family protein, which is a putative translation factor involved in translation, ribosomal structure, and biogenesis in Shewanella oneidensis and in the psychrophile Colwellia psychrerythraea 34H (Heidelberg et al. 2002; Methé et al. 2005); it is also essential for translation regulation in yeast (Lin et al. 2010). In Shewanella piezotolerans, the Sua5/YciO/YrdC/YwlC family protein has functions related to the assembly of ribosomes under low temperature conditions (Li et al. 2008b).

LOC_Os10g40934 is the gene that encodes a putative flavonol synthase, flavone 3-hydroxylase, or 2OG-Fe(II) oxygenase. Flavonols are present in plant parts that are colored, such as fruits and flowers (Mol et al. 1998). The accumulation of flavonoids has also been reported in Arabidopsis seeds (Routaboul et al. 2006). Flavonoids are effective antioxidant agents that can interrupt glucose and lipid metabolism by increasing glucose and lipid oxidation. Additionally, the relative compounds of flavonol, such as quercetin, help regulate auxin transportation, possess antioxidant properties, and are involved in signaling (Owens et al. 2008). Due to splicing, LOC_Os10g40934 has many transcript sizes. Therefore, in this study, we selected the two longest sizes to develop two types of overexpressing constructs of LOC_Os10g40934.3 (Os10, Os10 is the LOC_Os10g40934.3 splicing variant with a 645-bp CDS) and LOC_Os10g40934.11 (Os10L, has a larger transcript size than Os10; Os10L is the LOC_Os10g40934.11 splicing variant with a 1074-bp CDS) to transfer into Jatropha.

Initially, the transformation procedure for Jatropha was established via Agrobacterium tumefaciens infection of cotyledon explants using the herbicide phosphinothricin as the selection agent (Li et al. 2008a).
Subsequently, Kajikawa et al. (2012) developed a novel method of *Agrobacterium*-mediated transformation using the herbicide bispyribac-sodium; however, the explant regeneration efficiency was relatively low. The high efficiency of callus regeneration associated with *Agrobacterium*-mediated transformation at an early phase of regeneration, followed by shoot and root regeneration, is essential and could determine the success of the transformation.

In this study, we examined the favorable concentration and ratio of each phytohormone in Murashige and Skoog (MS) medium (Murashige and Skoog 1962) for Jatropha callus and shoot induction. When inducing root regeneration, we cultured shoot explants in Gamborg’s B5 medium (Gamborg et al. 1968) containing sucrose and 3-indolebutyric acid (IBA) to improve root induction.

Materials and methods

*Plant materials and media*

Wild-type Tanzania line *J. curcas* L. seeds (Sekisui Chemical Co., Ltd., Kyoto, Japan) were used as the transformation material; only the cotyledons inside the seeds were used for *Agrobacterium* infection. The infected explants, including the calli, shoots, and rooting plants, were incubated under controlled conditions with 16 h of light at 60 µmol/m²/s and 8 h of dark per day. The temperature and humidity were maintained at 26°C and 80%, respectively. MS medium was used as the basal formula for callus induction media (CIM) and shoot induction media (SIM). We found that MS medium with 1.0 mg/l 6-benzyladenine (BA) and 0.5 mg/l IBA was suitable for induction of callus formation, whereas MS medium with 3.0 mg/l BA and 0.1 mg/l IBA was suitable for induction of shoot formation (Enoki et al. 2017). For root induction media (RIM), we used Gamborg’s B5 as the basal formula instead of MS medium. In a previous study, we used MS medium as the basal medium for RIM, with some phytohormones added to the medium to improve the chances of inducing root formation; however, it did not improve root initialization and inhibited the cut shoots from producing roots. Gamborg’s B5 medium contains less NH₄ than MS medium and contains KNO₃, as the main source of nitrogen, which is reported to increase the percentage of root induction (Sriskandarajah et al. 1990). The plant phytohormones IBA (0.5 mg/l) and powder-dipped IBA were added to Gamborg’s B5 media and were used for a short period (3–7 days) to enhance root initialization. They were subsequently removed because the presence of phytohormones tends to hinder the rooting of cut shoots.

*A. tumefaciens* strains and vectors

*A. tumefaciens* strain EHA101 contains a C58 chromosomal background that is resistant to the antibiotic rifampicin; it also contains the pEHA101 Ti plasmid, which is resistant to the antibiotic kanamycin. *A. tumefaciens* strain EHA101 containing either vectors A2 or A3 (PalSelect system; Kumiai Chemical Industry Co. Ltd., Tokyo, Japan) was used. Vectors A2 and A3 are binary vectors that were used to transfer green fluorescent protein (GFP) gene and rice fl-cDNA into Jatropha. Both A2 and A3 contain the spectinomycin-resistance (SpR) gene as a selection marker for *Agrobacterium* and the *A. thaliana* acetolactate synthase (ALS) gene between the left border and right border regions as a selection marker gene for plants. The cloning sites of A2 and A3 are located between the left and right border: A2 has multiple cloning sites with various restriction sites, whereas A3 utilizes Gateway® cloning technology. The constructs to be transferred into Jatropha were prepared using Gateway® cloning technology (from pENTR/D-TOPO vector to A3 vector cloning, followed by LR reaction). The GFP gene was cloned into the A2 vector at multiple cloning sites, whereas the five rice fl-cDNAs were cloned into the A3 vector separately at the gateway cloning site.

*Agrobacterium culture and infection buffer preparation*

*Agrobacterium* containing the designed vectors was cultured in lysogeny broth liquid medium containing rifampicin (final concentration, 50 mg/l), spectinomycin (final concentration, 80 mg/l), and kanamycin (final concentration, 50 mg/l). Next, 10–14-h cell cultures of *Agrobacterium* were selected, and the optical density (OD) was measured at 600 nm to produce 15 ml of infection buffer with a final OD₆₀₀ of 0.2 using MS liquid medium and acetosyringone (final concentration, 100 µM).

*Explant preparation and Agrobacterium infection*

Jatropha seed shells were removed using pliers. The kernels were soaked in tap water for 8–14 h to soften the endosperm and allow water uptake to the cotyledons. The kernel endosperms were removed and cotyledons were collected. The collected cotyledons were dipped in a sterilization buffer (MilliQ water with 4% sodium hypochlorite and one drop of Tween 20) for 2 min. After sterilization, the cotyledons were washed with autoclaved water three–five times or until bubbles did not appear. The cotyledons were placed on a wet paper or inside a falcon tube to prevent drying and were cut into small pieces using a sterilized blade on a glass petri dish (one pair of cotyledons was cut into 12–16 small pieces of explants). The embryo was discarded because plants regenerated from the embryo are likely to resist the antibiotics that were used for the selection of the transgenic plants (escaping plants). The explants were dipped into the infection buffer and then placed inside an aspirator and vacuumed at −50 kPa for 5 min. Infected explants were transferred to the co-cultivation agar medium supported by filter paper and incubated at 26°C under dark conditions for 4 days.

*In vitro tissue culture of J curcas L*

After incubation for 4 days, the infected explants gradually changed into yellowish curling explants. Only yellow and curled explants were selected and transferred to a new
medium for sequential cultivation in CIM, SIM, RIM, and soil acclimation. The infected explants were cultured in CIM for 2 weeks, and then the calli were transferred to SIM. Shoots that emerged from the calli with a length of more than 10 mm were cut and transferred to RIM. Regenerated shoots from the calli were treated in RIM in the presence or absence of IBA (0.5 mg/l) under light (16 h of light at 60 µmol/m²/s and 8 h of dark) or dark conditions (24 h of dark) for 3 or 7 days before subculturing back to RIM without auxin. Dark incubation involved placing the shoot, after being cut from the callus, on RIM containing 0.5 mg/l IBA, which was then placed in an incubator without light for 3 or 7 days. Light incubation involved shoot cultivation under normal light conditions. Auxin was applied to the shoot by either directly dipping the shoot that was cut from the callus into the auxin powder mixture (Oxyberon; Bayer Cropscience, Leverkusen, Germany: IBA mixture) before placing it on basal RIM or the shoot was transferred to RIM containing 0.5 mg/l IBA. Next, the shoots were incubated in dark or light conditions for 3 or 7 days before being transferred again to basal RIM. Healthy rooted plantlets were transferred to sterile soil (autoclaved vermiculite and soil at a ratio of 1:1) and incubated under controlled conditions with 16 h of light at 60 µmol/m²/s and 8 h of dark per day (Figure 1).

Selection of transgenic plants using bispyribac-sodium

Infected explants were transferred from the co-cultivation medium to the selection medium using 20 nM bispyribac-sodium as a selection agent. Some of the calli from the co-cultivation medium were moved to CIM containing 20 nM bispyribac-sodium for the selection of resistant calli (delayed selection treatment), whereas the other calli obtained from the co-cultivation medium were moved to CIM without bispyribac-sodium for 2 or 4 weeks (delayed screening treatment) before being moved to CIM or SIM containing 20 nM bispyribac-sodium. The tolerance of the plant to the selection marker should be considered because young and mature explants have different antibiotic tolerances. We delayed the selection of transgenic plants by adding bispyribac-sodium to the media at the following time periods: the beginning of the culture after co-cultivation of the explant, 14 days after transformation, and 28 days after transformation (Table 3).

PCR-based analysis for screening candidate transgenic plants

Four types of primers were employed to screen candidate transgenic plants (see Supplementary Table 1 for primer sequences). DNA was extracted from 100 mg (fresh weight) of young leaf tissue from growing plants using the DNeasy® Plant mini kit (QIAGEN, Hilden, Germany) according to the kit protocols. The JcActin gene was used as a control and the ALS gene was used for selection by bispyribac-sodium from the A2 and A3 vectors. GFP, rice fl-cDNA, and the heat-inducible transcription repressor (HrcA) gene were used to detect possible contamination of Agrobacterium DNA in the extracted DNA (if the DNA samples of the young leaves of these plants showed HrcA expression, the DNA of different young leaves were extracted again to confirm the expression of the transferred gene) via amplification by PCR using RBC Taq DNA polymerase (RBC Bioscience, New Taipei, Taiwan). The PCR reaction mixture was created according to the kit instructions using 90 ng of extracted DNA and the following procedure: initial denaturation at 94°C for 2 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 94°C for 30 s, annealing for 1 min (at the temperatures listed in Supplementary Table S1), and extension at 72°C for 1 min; and final extension at 72°C for 7 min.

Expression of transferred genes

The expression of transferred genes in the transgenic plants was analyzed by the detection of transcripts using RT-PCR. Total RNA was extracted from 100 mg (fresh weight) of young leaf tissue obtained from growing transgenic plants using the RNeasy® Plant mini kit (QIAGEN, Hilden, Germany),
according to the kit protocols, with a modification using the cetyltrimethylammonium bromide extraction method (Sangha et al. 2010). The JcActin gene served as a control, and GFP and rice fl-cDNA were used to detect the expression of the transferred genes, which were amplified by RT-PCR with two types of primers (see Supplementary Table S2 for primer sequences) using the Prime Script High Fidelity RT-PCR Kit (TaKaRa, Shiga, Japan). PCR was performed as follows: 30 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 5 s, and extension at 72°C for 1 min.

**Acclimation and cultivation of transgenic plants**

Transgenic plants that expressed the transferred genes, as confirmed using PCR and RT-PCR, were grown in a sterile soil mixture of vermiculite and soil. Wild-type Tanzania Jatropha plants and transgenic plants were kept inside a specific netted house for the genetic engineering quarantine experiment at the University of Tsukuba. The fruits and seeds from transgenic plants were collected, and their width, length, thickness (volume was calculated using width, length, and thickness data using the ellipsoid shape formula: \( V = \frac{4}{3}\pi a b c \), where \(a\) is the width, \(b\) is the length, and \(c\) is the thickness), and fresh weight were measured.

**Ploidy levels of transgenic plants**

The transgenic plants, confirmed using PCR and RT-PCR, were further analyzed using a Beckman Coulter Gallios flow cytometer and software v.1.1 (Beckman Coulter, Inc., California, USA) to verify the ploidy levels of each plant.

**Results**

**Explant developmental process**

The regeneration of infected explants was classified into the following four progressive stages: infected explant, callus, shoot, and rooted plant. Infected explants were curled and initiated callus formation at 4 days post infection (DPI). The callus was enlarged and differentiated into primordial shoots within 21–84 DPI. Regenerated shoots were cut and placed into either RIM or the auxin root induction treatment. Depending on the treatment, regenerated roots were noted between 91 and 147 DPI.

The length of the newly infected explants that had been incubated on co-cultivation media was assessed starting at 1 day and up to 7 days. From day 4 onward, almost all infected explants had fully developed into the second stage, called a callus. On the other hand, from day 6 onward, some of the developed callus were oozing Agrobacterium, which worsened with time.

The developed callus were large, and two different types of calli were identified. The first type was a rough and bumpy callus; this type of callus was likely to produce shoots as the third stage of development. The second type of callus had a smoother surface and a sponge-like characteristic; it rarely produced any shoots. Regenerated shoots usually elongated up to 2 cm within 7 to 14 days. Shoots longer than 1 cm were transplanted into RIM to induce the shoot to progress to the fourth stage, a rooted plant. Shoots with adventitious roots were taken from RIM, residual medium was washed away, and they were transplanted into sterile soil media. At this stage, most of the rooted plants successfully produced root hair and functioned normally like germinated plants.

**Auxin-improved root regeneration**

The root regeneration percentage for RIM (Gamborg's B5 formula) was relatively low (9.91% ± 9.60%) compared with that for RIM containing auxin (IBA; 33.42% ± 9.11%). This suggests that the addition of auxin to RIM promotes root regeneration from the cut shoot.

The powder-dipped auxin treatment and the RIM containing auxin (IBA) treatment had a greater effect on the root regeneration percentage of Jatropha shoots than the treatment of RIM without auxin (IBA; Table 1).

**Shoot culture in dark and light conditions affect root regeneration**

Shoots were incubated at different dark and light conditions to investigate their effects on root regeneration. Dark incubation led to noticeably higher root regeneration percentages (29.40% ± 11.70%) than light incubation (14.31% ± 12.21%).

The 3-day dark condition appeared to induce the highest root regeneration percentage (35.20%), followed by the 7-day dark condition (27.62%) and the light condition (14.31%; Table 2).

**Bispyribac-sodium selection delays improved in vitro Jatropha tissue culture**

The shoot regeneration percentages were not affected by the timing of bispyribac-sodium treatment (13.11–14.88%);

<table>
<thead>
<tr>
<th>Auxin treatment</th>
<th>Root regeneration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Powder-dipped and placed on root induction media (Oxyberon: IBA mixture)</td>
<td>34.38 ± 11.15&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Root induction media containing auxin 0.5 mg/l (IBA)</td>
<td>26.01 ± 11.38&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Root induction media</td>
<td>9.55 ± 8.67&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Each replicate comprised more than 30 separate explants. Experiments included three–five replicates. Average rooting and errors were calculated using SPSS software by comparing means method using one-way analysis of variance with a significance level of 95%. Data with different superscript letters are significantly different (Duncan's test at \( p \leq 0.05 \)).
Table 3). Conversely, root regeneration was affected by the timing of treatment, in which postponement of selection until after 28 days of transformation led to the highest percentage of rooted plantlets (42.19%), followed by selection at 14 days (25.58%), with the lowest percentage of rooted plantlets noted when selection was performed at the beginning of the culture (17.02%; Table 3).

**Transgenic plant detection and selection efficiency**

A total of 242 rooted plants were evaluated to determine whether they were transgenic; 15 plants were *Agrobacterium*-free transgenic plants, 6 were false-positive transgenic plants from *Agrobacterium* contamination, and 227 were false-positive. The efficiency of transgenic plant production using our method ranged from 2.78 to 10.53%, with a total efficacy of 6.20% (Table 4).

**Expression of transferred genes in transgenic lines**

The expression of the transferred genes was examined in the transgenic plants; each transgenic plant showed expression of the correct target gene (Figure 2 and Supplementary Figure S1).

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Table 2. Effect of growing conditions on Jatropha root regeneration.

<table>
<thead>
<tr>
<th>Growing condition</th>
<th>Root regeneration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 days of dark condition and then light condition</td>
<td>35.20±14.13a</td>
</tr>
<tr>
<td>7 days of dark condition and then light condition</td>
<td>27.62±7.02a,b</td>
</tr>
<tr>
<td>Light condition</td>
<td>14.31±12.21b</td>
</tr>
</tbody>
</table>

Each replicate comprised more than 30 separate explants. The experiment included three-five replicates. The average rooting and errors were calculated using SPSS software by the comparing means method using one-way analysis of variance with a significance level of 95%. Data with different superscript letters are significantly different (Duncan’s test at p≤0.05).

Table 3. Regeneration of explants, according to stage, as a response to different selection times.

<table>
<thead>
<tr>
<th>Selection timing</th>
<th>Number of calli</th>
<th>Number of shoots (%)</th>
<th>Number of rooting plants (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Start of study</td>
<td>353</td>
<td>47 (13.31)</td>
<td>8 (17.02)</td>
</tr>
<tr>
<td>14 days after transformation</td>
<td>328</td>
<td>43 (13.11)</td>
<td>11 (25.58)</td>
</tr>
<tr>
<td>28 days after transformation</td>
<td>430</td>
<td>64 (14.88)</td>
<td>27 (42.19)</td>
</tr>
</tbody>
</table>

Table 4. PCR-based screening of rooting plants.

<table>
<thead>
<tr>
<th>Explant</th>
<th>Number of rooting plants</th>
<th>Number of transferred gene positive samples (%)</th>
<th>Number of transferred gene false-positive samples from <em>Agrobacterium</em> DNA (%)</th>
<th>Number of <em>Agrobacterium</em>-free transgenic plants (%)</th>
<th>Number of false-positive plants not containing the transferred gene (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sGFP</td>
<td>32</td>
<td>6 (18.75)</td>
<td>3 (9.38)</td>
<td>3 (9.38)</td>
<td>29 (90.63)</td>
</tr>
<tr>
<td>Os03</td>
<td>42</td>
<td>3 (7.14)</td>
<td>1 (2.38)</td>
<td>2 (4.76)</td>
<td>40 (95.24)</td>
</tr>
<tr>
<td>Os04</td>
<td>53</td>
<td>3 (5.66)</td>
<td>—</td>
<td>3 (5.66)</td>
<td>50 (94.34)</td>
</tr>
<tr>
<td>Os08</td>
<td>36</td>
<td>1 (2.78)</td>
<td>—</td>
<td>1 (2.78)</td>
<td>35 (97.22)</td>
</tr>
<tr>
<td>Os10</td>
<td>38</td>
<td>6 (15.79)</td>
<td>2 (5.26)</td>
<td>4 (10.53)</td>
<td>34 (89.47)</td>
</tr>
<tr>
<td>Os10L</td>
<td>41</td>
<td>2 (4.88)</td>
<td>—</td>
<td>2 (4.88)</td>
<td>39 (95.12)</td>
</tr>
<tr>
<td>Total</td>
<td>242</td>
<td>21 (8.68)</td>
<td>6 (2.48)</td>
<td>15 (6.20)</td>
<td>227 (93.80)</td>
</tr>
</tbody>
</table>

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Figure 2. RT-PCR analysis of the transferred genes in transgenic Jatropha plants. a. Amplification of sGFP gene. b. Amplification of LOC_Os03g49180 (Os03) gene. c. Amplification of LOC_Os04g43210 (Os04) gene. d. Amplification of LOC_Os08g41910 (Os08) gene. e. Amplification of LOC_Os10g40934 (Os10) gene. f. Amplification of LOC_Os10g40934.11 (Os10L: larger transcript size than Os10) gene. Lanes: bp, molecular size marker; W, wild-type plant; Number refers to independent transgenic plants.
Ploidy level of transgenic Jatropha plants
Analysis via flow cytometry revealed that all transgenic plants were diploid (Figure 3).

Seed size of transgenic Jatropha plants
Transgenic plants were grown in a controllable greenhouse; however, they hardly produced flowers or seeds; only 1 out of 15 transgenic lines (Os10-4 plant: the transgenic plant of LOC_Os10g40934.3) produced seeds. The original wild-type seeds from Tanzania were larger and heavier than those of wild-type Tanzania Jatropha plants grown in Japan, with 2–4 times the seed volume and 1.5–2 times the seed weight (Table 5). The seeds of transgenic Os10-4 plants were larger and heavier than those of wild-type Tanzania Jatropha plants grown in Japan, with almost twice the seed volume and 1.5 times the seed weight.

Discussion

Auxin application increases root induction efficiency
Jatropha in vitro tissue culture and transformation were performed to determine their most optimal growth conditions. Many factors affect explant regeneration; these can be both biological factors, such as the genetics of the plant, the explant condition, or the vector strain, and nonbiological factors, such as the phytohormone combination, incubation environment, or transformation procedure (Kalimuthu et al. 2007; Kumar and Reddy 2010; Sharma et al. 2011; Singh et al. 2010; Sujatha and Dhingra 1993; Sujatha and Mukta 1996; Sujatha et al. 2005). This study identified some factors that improve the likelihood of successful Jatropha transformation, particularly at the most critical point, i.e., the induction of root regeneration.

Adventitious root formation from excised shoots is promoted by auxin, the main component in many root-inducing products. The cut shoot is unique in that allows the adventitious root to form more efficiently than normal, particularly under conditions of altered nutrition resources and endohormone homeostasis (Druege et al. 2019). Explants cultured in RIM and auxin had a three-times higher percentage of root regeneration than explants with no-auxin treatment, suggesting that auxin is indeed crucial for Jatropha adventitious root regeneration. Rooting may be induced by condensed auxin, which is normally synthesized in the shoot tip and transported to the base of the shoot, where it accumulates, rather than being transported to the calli, in addition to auxin uptake from the media. The effect of auxin on root induction has been investigated both in vitro (Purkayastha et al. 2010) and in vivo (Camellia et al. 2009). The role of auxin may be related to the adventitious rooting of plants over hormone cross-talks (Pacurar et al. 2014). For instance, genes involved in auxin homeostasis are widely differentially expressed in cuttings of tea (Camellia sinensis) with and without IBA treatment (Wei et al. 2019). Moreover, auxin and auxin-derivative compounds such as melatonin can promote a stronger effect on the rooting of plants (Sarropoulou et al. 2012).

Dark condition and auxin application
As shown in Table 2, shoots that were incubated in the dark for 3 days had a greater chance of root formation than those incubated in the dark for 7 days, but the shoots that continued to be incubated in the dark

Table 5. Size and weight of wild-type Tanzania Jatropha seeds, wild-type Tanzania Jatropha seeds grown in Japan, and Os10-4 transgenic Jatropha seeds.

<table>
<thead>
<tr>
<th></th>
<th>Seed width (mm)</th>
<th>Seed length (mm)</th>
<th>Seed thickness (mm)</th>
<th>Seed volume (mm³)</th>
<th>Seed weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original wild-type seeds from Tanzania</td>
<td>12.28±0.58</td>
<td>19.02±0.05</td>
<td>9.61±0.45</td>
<td>1176.46±85.88</td>
<td>0.74±0.06</td>
</tr>
<tr>
<td>Seeds from wild-type Tanzania planted in Japan</td>
<td>7.12±0.28</td>
<td>14.95±0.32</td>
<td>6.73±0.65</td>
<td>374.53±40.03</td>
<td>0.31±0.04</td>
</tr>
<tr>
<td>Seeds of transgenic Os10-4 plant</td>
<td>10.84±0.18</td>
<td>16.21±0.44</td>
<td>8.46±0.14</td>
<td>779.18±41.48</td>
<td>0.58±0.03</td>
</tr>
</tbody>
</table>

Data with different superscript letters are significantly different (Duncan’s test at p≤0.05).
Transformation of *Jatropha* for production of larger seeds

gradually lost their green color from 7 days onward. This result suggests that a short period of darkness is important for root formation and regeneration. A study of rice adventitious roots from the stem found that the dark condition is necessary and improves adventitious root formation through auxin transportation and ethylene-related growth promotion (Lin and Sauter 2019). In petunias (*Petunia* hybrida), dark incubation accelerates the root formation associated with the accumulation of indole-3-acetic acid in the base of the stem (Yang et al. 2019). In *Jatropha*, auxin improves the rooting quality of the cut stem (Camellia et al. 2009); however, for in vitro culture, the effect of auxin on root formation in *Jatropha* has not yet been investigated.

In this study, we examined the effects of IBA and dark conditions on *Jatropha* root induction. The IBA and dark treatments led to a higher percentage of rooting than the light treatment (Tables 1, 2), suggesting that dark treatment applied to the in vitro tissue culture of *Jatropha* can improve its rooting efficiency. The 3-day treatment led to a higher percentage of rooting than the 7-day treatment. These results may be due to the morphology of *Jatropha*. Regenerated shoots usually had one–three leaves, which were small at the root induction stage. When shoots are incubated in the dark condition, photosynthesis cannot occur, and the shoots gradually reduce energy production. Therefore, even though adventitious root regeneration can be promoted by a carbon source in the shoot area, the reserved energy inside the cells cannot be replenished, resulting in a lower rooting percentage in the 7-day treatment. These data suggest that a short dark period facilitates the initialization of root regeneration in *Jatropha*.

**Delayed bispyribac-sodium selection**

As expected, the number of regenerated shoots and rooting plants increased with delays of the selection marker. Explants in the cotyledon phase may have lower viability than callus and shoot explants; therefore, the survivability of explants may be improved when screening begins at a later explant phase. Similar experiments have been conducted using kanamycin as a selection marker (Fu et al. 2015); however, the results demonstrated that growing explants without kanamycin for 1 week could produce more regenerated shoots than growing explants without kanamycin for 2 or 3 weeks. We assume that the difference in the selection mechanism of bispyribac-sodium and kanamycin leads to a difference in the timing of screening and that including all plant samples can also affect the results.

**Transgenic plant efficiency**

The *Agrobacterium*-free transgenic plants were estimated as roughly one true transgenic plant for every 6–36 rooting plants, with a high number of false positives. Nonetheless, the high number of rooting plants suggests a good protocol, but the selection for true transgenic plants requires more attention as the number of false-positive plants also increases. Nevertheless, we still strongly believe that the chances of producing successful transgenic plants, even when there are many false-positive plants, is better than if strictly selecting using the selection marker with the loss of transgenic plants. Notably, we noted a case of a transgenic sGFP control shoot that never produced any roots; hence, we could not transfer the plants into the soil.

**Transgenic plant characteristics**

The transcription and expression of both endogenous and exogenous genes can affect the productivity of the plant. We investigated the expression of transferred genes to confirm that the transferred genes were properly expressed.

Inducing polyploidy in *Jatropha* is a widely employed strategy to produce larger seeds for increased biofuel production (de Oliveira et al. 2013; Niu et al. 2016; Premjet et al. 2019). Some polyploid *Jatropha* lines have vegetative parts with different sizes and characteristics as well as different fruits and seeds due to changes in their genetic material. Our transgenic plants were confirmed to be diploid plants; thus, we can conclude that the results cannot be due to the effect of polyploidization.

The transgenic *Jatropha* plants hardly produced flowers. Only one transgenic plant managed to produce fruits and seeds: the Os10-4 plant that expressed the rice LOC_Os10g40934 gene. Transgenic Os10-4 plant seeds were smaller than those from Tanzania plants. Given that similar plants grown in Japan also produce seeds smaller than those produced by Tanzania plants, the environment in Japan may affect fruit and seed production. On the positive side, the transgenic seeds produced by the Os10-4 plant were larger than those produced by the wild-type plant grown in Japan, which suggests that our transgenic plants could fulfill the objectives required for biofuel production.

In the present study, we successfully applied our modified methods to create transgenic *Jatropha* plants that had a higher chance of survival than those produced in past studies, mainly by improving the induction of roots from shoot explants. Additionally, we found an interesting effect of auxin application and dark incubation for in vitro *Jatropha* tissue culture. Our method is simple, practical, and can be used in most laboratories. In the future, we hope that our transgenic plants will eventually produce fruits and seeds; these could be valuable resources for biofuel and plant lipid research as well as for the production of green energy to mitigate the issues related to global warming.
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