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Agrobacterium-mediated transformation of *Jatropha curcas* L. with a polyhydroxyalkanoate gene (*phaC*)

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ABSTRACT Polyhydroxybutyrate is a component of bioplastics that is synthesized under the control of enzymes encoded by *pha* multigenes. The genes are naturally present in *Ralstonia eutropha*. However, the production of bioplastics in bacteria is inefficient because the bacterial biomass is relatively small compared with plants or fungi. As such, engineering techniques have been developed that enable *pha* genes to be inserted into plant biomass, and then be expressed in the biomass of the plant to produce polyhydroxybutyrate. The objectives of this study were to transform the tissue of *Jatropha curcas* using the *phaC* gene (a *pha* gene), to regenerate the transformed plant, and to confirm the presence of the inserted genes with PCR. The genetic transformation of *J. curcas* was mediated by *Agrobacterium tumefaciens* strain GV3101 containing pARTC by dipping the cotyledon tissue of *J. curcas* in a suspension of the bacterium for 30 min, followed by cocultivation for 3 d on Murashige and Skoog (MS) medium. The tissue was then placed on a selection medium, i.e. MS medium containing 13.3 µM BAP and 0.05 µM IBA with the addition of 20 mg/L kanamycin. The results showed that 12.35% of the tissue survived and regenerated into a shoot after 1–2 months. Molecular analysis of the transformed tissue was performed using *phaC* and *nptII* primers, in order to detect the presence of the *phaC* and *nptII* genes. Specific bands were detected at 659 bp and 700 bp, corresponding to the *nptII* primer and *phaC* primer, respectively.

KEYWORDS *Agrobacterium tumefaciens*; bioplastic; *Jatropha curcas*; *nptII*; *phaC*

1. Introduction

Bioplastic is a plastic material derived from renewable energy sources of living organisms and is biodegradable. Some of these materials obtained from microbial activity on plant material such as vegetable oil, corn starch and legumes. The main component of bioplastics is polyhydroxybutyrate (PHB) which resemble the components in petroplastics polypropylene.

PHB is a compound derived from polyhydroxyalkanoate (PHA) which is one of renewable resources-based polymers of bioplastic (Reddy et al. 2003). PHB is synthesized from two acetyl coenzim-A in the presence of three enzymes β-ketothiolase, acetoacetyl-CoA reductase and PHA synthase which encoded by multiple genes of *phaA, phaB, phaC*, respectively (Lössl et al. 2003). Those three genes are naturally found in bacteria *Ralstonia eutropha* that responsible in carbon storage (Poirier 2002). Therefore, existing bioplastic industry uses culture of *R. eutropha* to convert sugar plant into PHB.

The PHB production by using culture of bacteria encounters some obstacles, i.e. low productivity and higher cost compared to that production by using petroplastic polypropylene. This because of the bacterial biomass is relatively small. Therefore it is necessary to look for larger organisms that can express the three genes to produce more quantities of PHB. With genetic engineering techniques it is allowed *pha* genes from *R. eutropha* are isolated and inserted into plant which can then be expressed in the biomass of the plant to produce PHB.

Studies regarding PHB production on transgenic plants has been developed in several plants. Poirier et al. (1992) inserted gene encoding PHB in *Arabidopsis thaliana* that produced 0.1% PHB in its dry biomass. Lössl et al. (2003) conducted transformation of PHB-related genes in *Nicotiana tabacum* which obtained 1.7% PHB in its dry biomass. *A. thaliana* was reported to produce 40% PHB in dry weight of leaves (Bohmert et al. 2002). Moreover, PHB expression as much as 0.36% was reported in shell of transgenic soy bean seed Schnell et al. (2012).

With regard to the matter discussed above, we were interested to create plants producing bioplastic material by doing genetic transformation that is by inserting PHB genes into plant tissue. One of the good candidates

plant for this purposes was *Jatropha curcas*, a non-edible plants producing seeds that belongs to Euphorbiaceae, and widely known to produce oil for biodiesel due to abundant oil content of its seed. *J. curcas* is unable to produce PHB because it does not have any genes for PHB synthesis. On the other hand, this plant produces a lot of seeds that contain high acetyl-CoA which is responsible as substrate in PHB synthesis, so that *J. curcas* is very suitable to be genetically engineered to contain PHB genes for then to be able to produce PHB in their seed.

Previous study reported the isolation of *phaC* gene and construction of *phaC* gene together with *nptII* gene as marker gene in plant expression vector pARTC (Widhiasi 2011). This vector composed of seed specific promoter *LKfCS3*, peptide signal *ctp*, *phaC* gene and *nos* terminator, that can be expressed in plants. *LKfCS3* seed specific promoter plays a role to promote PHB synhtesis in seed without affecting transgenic plants growth.

There are several reports of *A. tumefaciens*-mediated in *J. curcas*. Kajikawa et al. (2012) succesfully transformed *J. curcas* using *A. tumefaciens* and found the shoot frequency of cotyledon explant was higher than hypocotyl explant. Pan et al. (2010) and Khemkladngoen et al. (2011) also succesfully transformed *J. curcas* using *A. tumefaciens* with cotyledon explant.

However, there was less report on the transformation of *J. curcas* with PHB genes. Therefore, the objectives of present study were to transform tissue of *J. curcas* by *phaC* gene (one of *pha* genes), to regenerate transformed plant (*J. curcas* containing *phaC* gene), and to confirm the presence of inserted genes by PCR technique. Genetic transformation was performed in *J. curcas* using pARTC vector mediated by *A. tumefaciens*.

2. Materials and methods

2.1. Plant materials

The explant used in this study was cotyledon obtained from *J. curcas* seed IP-3P aged 7 days. The seeds were obtained from the Research Institute for Tobacco Plant and Fiber, the Plantation Research and Development Center (*Balai Penelitian Tanaman Tembakau dan Serat, Pusat Penelitian dan Pengembangan Perkebunan*), Bogor, Indonesia. To obtain cotyledon, the seeds were washed with water for 30 min, and further washed with detergent for 30 min, and alcohol 70% for 5 min. The seeds were peeled and washed with 30% solution of 5.25% natrium hypochlorite (NaClO) for 15 min and 15% solution of 5.25% NaClO for 10 min, and blot-dried on sterile filter paper, were further cut into two pieces vertically and placed in solid free Murashige & Skoog (MS) medium to allow germination. After 7 d of incubation, the emerged cotyledons were cut into pieces approximately 1 cm in size, then planted in pre-culture solid MS medium with 13.3 µM BAP and 0.05 µM IBA for 3 d, prior to *Agrobacterium*-mediated transformation.

2.2. Agrobacterium strain, plasmid and culture conditions

The expression vector pARTC (Figure 1) with kanamycin selection and containing *phaC* cassete (promoter *LKfCS3*-peptide signal *ctp*-*phaC* gene-*nos* terminator) was introduced into *A. tumefaciens* strain GV3101 and used for stable transformation. A single colony of *A. tumefaciens* strain GV3101 containing pARTC expression vector was cultured in 5 mL liquid YEP in addition of rifampisin 25 mg/L and kanamycin 50 mg/L. Culture was further planted for 3 d with agitation of 250 rpm, at 25°C and dark condition. One percent of liquid culture was replaced to 50 mL YEP in addition of rifampicin 25 mg/L and kanamycin 50 mg/L to obtain OD_{600} = 0.6. Suspension of *A. tumefaciens* was transferred to tube and centrifuged at 4000 rpm for 20 min. Supernatant was removed and pellet of *A. tumefaciens* was further solved in liquid half-strength MS in ratio 1:3. Suspension was used in *A. tumefaciens* infection for transformation.

2.3. Genetic transformation and plant regeneration of *J. curcas*

Tranformation by *A. tumefaciens* was applied into cotyledon explants of *J. curcas* by dipping method. Firstly, cotyledon explants were soaked for 30 min in suspension of *A. tumefaciens* containing 100 µM acetosyringone while shaken at 100 rpm. Afterwards, explants were blot-dried on sterile paper and planted on cocultivation solid MS medium supplemented with 13.3 µM BAP, 0.05 µM IBA and 100 µM acetosyringone for 3 d in the dark at approximately 25°C. The explants werethen washed three times with sterile water for 5 min and two times with Augmentin™ 300 mg/L solution for 10 min to eliminate ex-

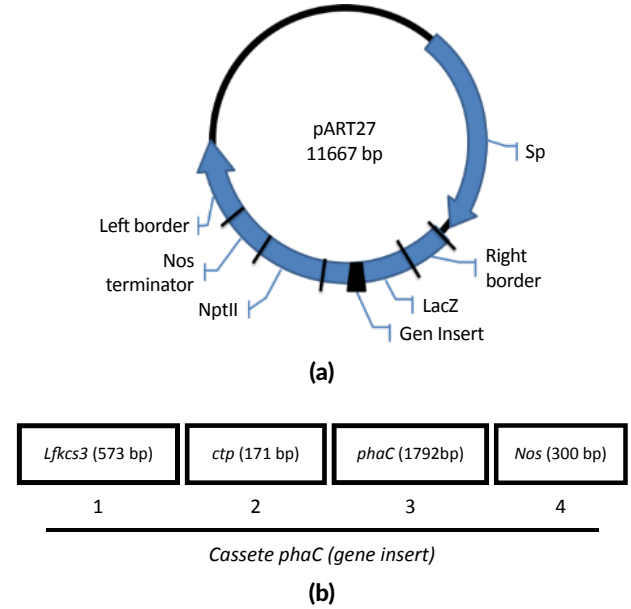

FIGURE 1 Plasmid pARTC expression vector. (a) pARTC map. (b) cassete of *phaC*: (1) promoter *Lfkcs3* (573 bp); (2) peptide signal *ctp* (171 bp); (3) *phaC* gene (1792 bp); (4) terminator *nos* (300 bp).

cess of bacteria. Washed explants were blot-dried on sterile paper and transferred to selection medium consisted of solid MS medium supplemented with 13.3 µM BAP, 0.05 µM IBA, 300 mg/L Augmentin™ and 20 mg/L kanamycin according to method of Pan et al. (2010) for 4–8 weeks.

Survived explants were sub-cultured into shoot inducing medium, which consisted of solid MS medium supplemented with 13.3 µM BAP, 0.05 µM IBA, 150 mg/L Augmentin™ and 20 mg/L kanamycin. Furthermore, shoots were transferred into elongation medium, which consisted of solid MS medium with 1.0 µM IBA and Augmentin™ 150.0 mg/L. Shoots were further transferred into rooting medium, a liquid half strength MS medium supplemented with 4.9 µM (IBA). All media were adjusted to pH 5.6–5.8, solidified with 0.8% (w/v) agar, and autoclaved at 121°C for 15 min. Antibiotics were filter-sterilized prior to being added into 40–50°C cooling autoclaved medium.

2.4. Molecular analysis of A. tumefaciens colony and transgenic plants

The presence of *nptII* and *phaC* in the T-DNA was examined in colony of *A. tumefaciens* (crude PCR) and putative transformant with *nptII* and *phaC* primers. The DNA template of *A. tumefaciens* was obtained by suspending single colony in 10 µL sterile deionized water. Whereas genomic DNAs were isolated from shoot of putative transformant and non-transgenic controls using the cetyl trimethylammonium bromide (CTAB) methode (Doyle 1990).

KAPA2G Fast PCR Kit (KAPA Biosystems) used to confirm *nptII* gene with primer: *nptII*_reverse (5-CCT TAT CCG CAA CTT CTT TAC CTA-3') and *nptII*_forward (5-ACA CCC AGC CGG CCA CAC AGT CG-3'). The protocol for PCR reaction as follow: initial denaturation of 95°C for 3 min, 35 cycles of denaturation at 95°C for 15 s, annealing 55°C for 15 s, elongation 72°C 15 s and final elongation at 72°C for 1 min. Confirmation of *phaC* was performed with Dream Taq PCR Kit (Thermo Scientific) using *phaC_forward* (5'-TCT AGA AAC ATG GCG ACC GGC AAA-3') and *phaC_reverse* (5'-ATC GCG AAG ATC TGA GTC ATG CCT TGG-3'). The protocol included as follow: initial denaturation at 95°C for 3 min, 30 cycles of denaturation at 95°C for 30 s, annealing 57°C for 30 s, elongation 72°C for 1 min and final elongation at 72°C for 5 min. PCR amplification products were separated on a 1.0% (w/v) agarose gels, stained with ethidium bromide and visualized under UV light.

3. Results and discussion

3.1. Molecular analysis of phaC gene in pARTC plasmid, nptII, and phaC genes in A. tumefaciens strain GV3101

The expression vector pARTC commonly used in plants that containing *phaC* as one of responsible genes in PHB biosynthesis. The presence of *phaC* in pARTC was initially confirmed prior to its transformation into *A. tumefaciens*. DNA amplification of pARTC using *phaC* primer resulted 1700 bp bands on lane 1–5 (Figure 2a) which corresponds to the gen *phaC* (1792 bp) as reported by Ayudia (2010) and Widhiasi (2011) that *phaC* gene had 1790s bp. Therefore, it was assumed that pARTC contained *phaC*, that involve in PHB synthesis. The T-DNA of pARTC was also contained nptII gene, a marker gene for kanamycin resistance. This plasmid was further transformed into *A. tumefaciens* strain GV3101 that used in this experiment as transformation vector in *J. curcas* tissue.

Transformation of pARTC into *A. tumefaciens* was confirmed using crude PCR with using *phaC* and *nptII* as the primers. Crude PCR analysis of *A. tumefaciens* that had been grown in 50 mg/L kanamycin by using a pair of *nptII* primers, resulted 659 bp bands on lane 1–

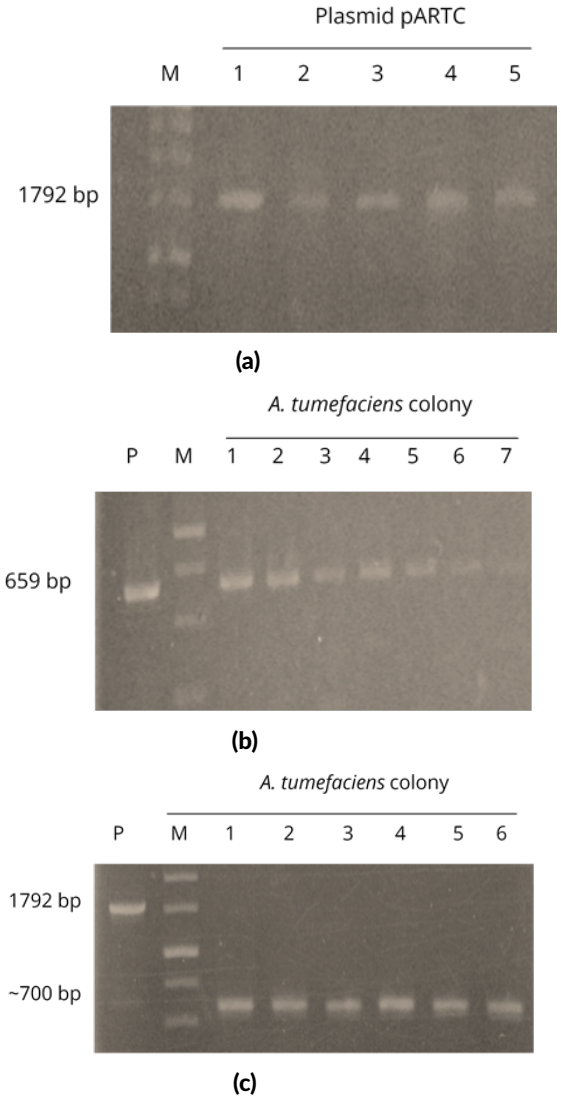

FIGURE 2 Confirmation the construct of pARTC plasmid. (a) confirmation of *phaC* gene in pARTC plasmid using *phaC* primer (lane 1–5); (b) confirmation of *nptII* gene of *Agrobacterium tumefaciens* colony using *nptII* primer (Lane 1–7); (c) confirmation of *phaC* gene of *A. tumefaciens* colony using *phaC* primer (Lane 1–6). P is pARTC plasmid as a positive control. M = 1 kb DNA marker.

7 and on lane P (Figure 2b). Lane 1–7 were colonies of *A. tumefaciens* transformed with pARTC, whereas lane P was pARTC used as a positive control. The *nptII* primers used in this study based on *nptII* primers designed by Khanna et al. (2004) to obtain PCR product of 659 bp. Electrophoregram showed that transformed *A. tumefaciens* strain GV3101 contained pARTC. The *nptII* gene was located in T-DNA of pARTC that was inserted to *A. tumefaciens* GV3101 and the gen was used as screening agent for both bacteria and plants. From this results, it was confirmed that the transformed *A. tumefaciens* strain GV3101 contained pARTC.

The colony of *A. tumefaciens* grown in a medium containing 50 mg/L kanamycin was also confirmed by referring to the presence of *phaC* using crude PCR with *phaC* primer. Electrophoregram showed bands 700 bp on lane 1–6 colonies of polyhydroxybutyrate (Figure 2c). Lane P which represent pARTC showed band 1792 bp when using *phaC* as the primer (Figure 2c). Electrophoregram of *A. tumefaciens* (lane 1–6, Figure 2c) which use *phaC* primer showed different results compare to the positive control of pARTC (lane P, Figure 2c). The logical explanation regarding the difference between pARTC (lane P) and DNA of transformed *A. tumefaciens* remains unclear. It might be caused by deletion some segment of DNA when pARTC was inserted into *A. tumefaciens*. To determine the cause of it, it is necessary to analysis the pcr product from genome of *A. tumefaciens* that has been inserted with pARTC carrying *phaC* gene by DNA sequencing. So that, whether the *phaC* gene has been completely inserted into T-DNA of *A. tumefaciens* or not can be determined.

According Bočkor et al. (2013), deletion or insertion can occur in bacteria. For example, the genetic analysis of *Eschericia coli* shows underlying mechanism in deletion or duplication which involves *RecA-dependent* and *RecA-independent*. *RecA-dependent* relies upon homolog recombination which causes different products of crossing between two DNA, resulting different DNA from its origin. Whereas, *RecA-independent* can generate error of DNA sequence alignment during replication. Both mechanisms are also found in *A. tumefaciens*.

3.2. Regeneration of transformed *J. curcas*

Transformed *J. curcas* could generate callus (Figure 3b) after 2–3 weeks incubation in selection medium, and further developed into shoot (Figure 3c) after 4–6 weeks incubation in shoot-inducing medium, which consisted of solid MS medium supplemented with 13.3 µM BAP, 0.05 µM IBA, 300 mg/L Augmentin™ and 20 mg/L kanamycin (Pan et al. 2010). These finding indicated that transformed *J. curcas* contained T-DNA which was carried by pARTC plasmid vector. On the other hand, untransformed *J. curcas* slowly underwent death in medium containing kanamycin 20 mg/L, which was indicated by alteration the color tissue to brown color (Figure 3f). This was happened

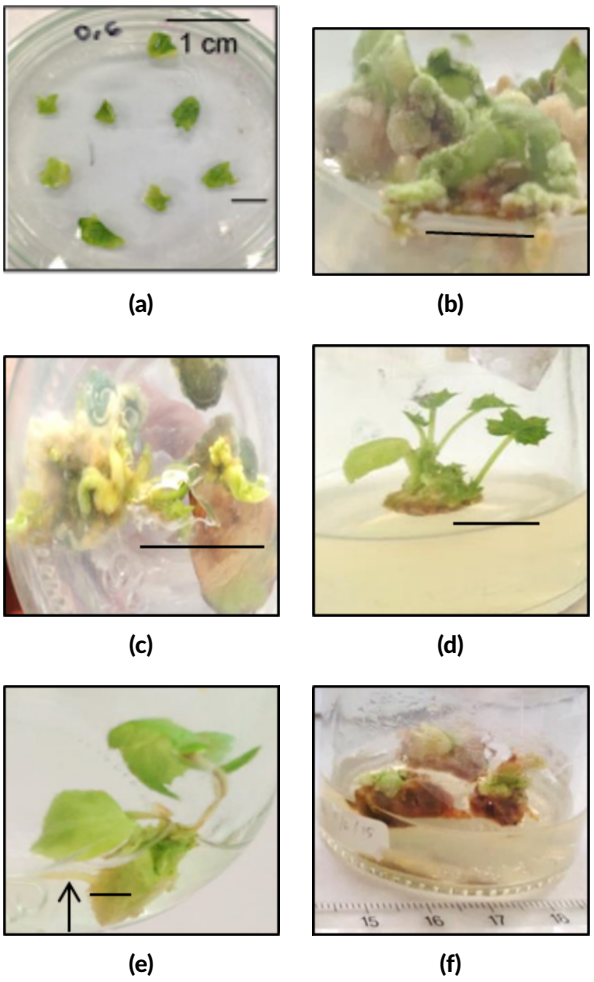

FIGURE 3 *Agrobacterium tumefaciens*-mediated transformation and regeneration of *Jatropha curcas* using cotyledon explants. (a) pre-cultured cotyledon explant; (b) calli induced on selective medium with Augmentin™ 300 mg/L and kanamycin 20 mg/L; (c) shoot induced on selective medium with Augmentin™ 300 mg/L and kanamycin 20 mg/L; (d) elongation shoot on elongation medium, which consisted solid MS supplemented with 1 µM IBA and 150 mg/L Augmentin™ without kanamycin; (e) rooted transgenic plant; (f) untransformed *J. curcas* on selective medium.

because untransformed tissue did not have *nptII* gene so that tissue not has resistance to kanamycin.

When shoot was transfered into elongation medium, which consisted of solid MS medium supplemented with 1.0 µM IBA and 150 mg/L Augmentin™ but without the addition of kanamycin, the shoots were well elongated (Figure 3d) and when the elongated shoots were transferred on root inducing-medium, which consisted liquid half-strength MS supplemented with 4.9 µM IBA and 150 mg/L Augmentin™, the plantlet of *J. curcas* was regenerated (Figure 3e). In this experiment, from two replications of transformation using 70 and 80 explants, percentage of *J. curcas* that able to generate shoots resistant to kanamycin was 12.35% (Table 1).

In this study, seen that regeneration of *J. curcas* from *Agrobacterium*-mediated transformation was obtained with indirect organogenesis, that was through cal-

TABLE 1 Percentage of *Jatropha curcas* transformation to form shoot.

Replication	Transformed explants	Regenerated explants forming shoot in kanamycin selective medium	Percentage (%)
I	70	8	11.40
II	60	8	13.30
Average			12.35

lus formation followed by shoot formation. The growth factors used, BAP and IBA, were responsible in the shoot regeneration. As reported by Khemkladngoen et al. (2011) that 13.3 µM BAP and 0.5 µM IBA could promote callus formation followed by shoot formation in *J. curcas* with growth effieciency as much as 78%.

Rooting induction of transformed *J. curcas* was rather difficult to be obtained, but nevertheless was successfully obtained when using liquid half-strenght MS with 4.9 µM IBA (Figure 3e). Similar finding was also reported by Toppo et al. (2012) and Kajikawa et al. (2012) that the use of indole butiric acid (IBA) as a growth factor gave a better result in the induction of root than indole acetic acid (IAA) as an endogenous auxin. Auxin induces root fomation by breaking root apical dominance through cytokinins.

Success rate was measured based on ratio between number of explants regenerated to shoot in selective medium and number of explants initially planted. Transformation of *J. curcas* was conducted in two repetitions using *A. tumefaciens* strain GV3101 with OD_{600} = 0.6 and soaked for 30 min. The average frequencies of *J. curcas* to generate shoot resistant to kanamycin in this study was 12.35% (Table 1), which was lower than the results obtained by Kumar et al. (2010), 29%, by using a similar bacterial density (OD_{600} = 0.6). Kumar et al. (2010) conducted transformation of *J. curcas* mediated by different *A. tumefaciens* strain, i.e. LBA 4404. The different used of strain of bacteria in this study might caused the lower frequencies of transformation.

3.3. Confirmation of J. curcas putative transformant using PCR method

The presences of *nptII* and *phaC* on *J. curcas* putative transformants were detected with PCR using *nptII* and *phaC* primers. The PCR product of *J. curcas* putative transformant using *nptII* primer was 659 bp (Figure 4a), indicating the integration of *nptII* in the *J. curcas* genome. Neomycin phosphotransferase II (*nptII*) gene plays a role to inactivated antibiotic from amino-glycosylated group such as kanamycin, genitisin and neomycin. The *nptII* gene is located in T-DNA as selective agent for transformed *J. curcas*.

PCR amplified DNA of *J. curcas* putative transformant using *phaC* primer was 700 bp (Figure 4b), as well as band of *A. tumefaciens* confirmed by using *phaC* primer (Figure 2c) to contain *phaC* gene. It indicates that *phaC* gene of the T-DNA of *A. tumefaciens* was integrated in plant genome. Thus, tranformation in this study took place accordingly. Yet, it does not confirm the presence of *phaC* (1792 bp) in tranformed plants genome by the *A. tumefaciens*. This might be due to the deletion or insertion in integration of T-DNA in *A. tumefaciens*. Further analysis is therefore needed, such as sequencing of PCR products using *phaC* primer.

Confirmation of *J. curcas* putative transformant using *nptII* primer, showed *nptII* was integrated in *J. curcas* genome, indicated by band of 659 bp in accordance with positive control, pARTC. On the other hand, untransformed *J. curcas* not indicate the presence of *nptII*. Confirmation of *J. curcas* putative transformant using *phaC* primer obtained band of 700 bp as well as *A. tumefaciens* (Figure 4b), yet it was different than result obtained from pARTC amplification using *phaC* primer. Untransformed *J. curcas* that confirmed using *phaC* primer also not obtained, indicating the absence of *phaC*. It might be caused by deletion of *phaC* in *A. tumefaciens* which may occurr during integration to plant genome.

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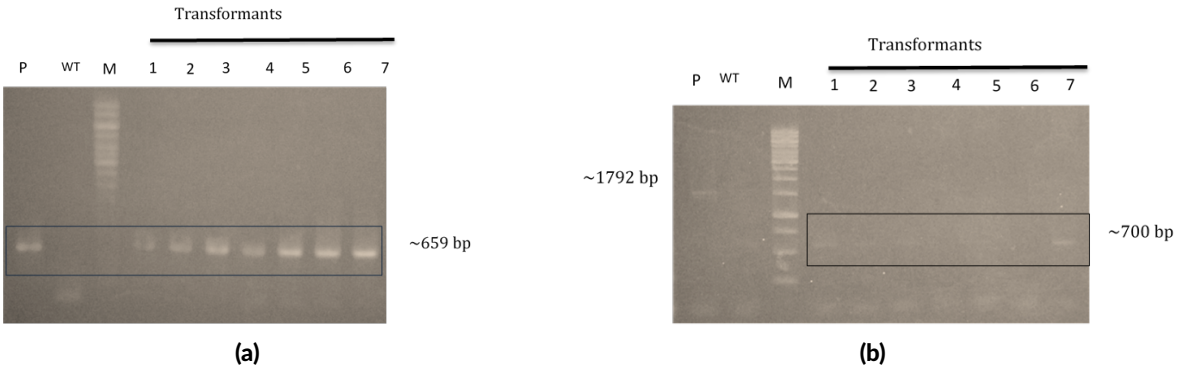

FIGURE 4 Molecular analysis of *Jatropha curcas* putative transformant by PCR method. (a) PCR analysis for detection *nptII* gene using primer *nptII*; (b) PCR analysis for detection *phaC* gene using primer *phaC*. Lanes M = 1 kb DNA marker gene. P = positive control (plasmid pARTC), WT = negative control (untransformed *J. curcas*), Lanes 1–7 = *J. curcas* putative transformants.

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Deletion or insertion can occurs during T-DNA integration to plant genome. Integration of T-DNA in plant genome remains elusive. Although T-DNA is believed to integrate to plant genome via DNA double stranded breaks (DSB) repair. Underlying mechanism of DNA double stranded breaks (DSB) repair in plants involve nonhomolog end joining (NHEJ). The presence of DSB will promote T-DNA binding to DSB, and later repaired via NHEJ. In this pathway, DNA repair is conducted without homolog sequence and directly ligated, usually followed by deletion or insertion on T-DNA (Tzfira 2003). Referring to study done by Tzfira (2003), plants encountering DSB induced by UV have higher number of integrated T-DNA compared to plants without induction. However, there was only 0.81% plants had completely integrated by T-DNA. Deletion was also detected in 1.13% plants, the rest was T-DNA integrated by deleted sequence on some base, or insertion during integration to plants genome (Tzfira 2003).

4. Conclusions

In conclusion, the cotyledon tissue of *J. curcas* was successfully transformed by *A. tumefaciens* GV3101 carrying pARTC plasmid vector that contain *nptII* gene (659 bp) for being resistance to kanamycin antibiotics and regenerate callus on selection medium. The putative transgenic callus was differentiated to form microshoot on Murashige & Skoog medium with addition of 13.3 µM BAP and 0.5 µM IBA. Rooting of the microshoot was obtained when the shoot was transferred on liquid half-strenght MS with 4.9 µM IBA to produce planlet. Molecular anaysis of the planlet indicated the presence of *nptII* gene (659 bp). However, the presence of *phaC* gene (1792 bp) that is for synthesis of a component of bioplastic, polyhydroxybutirate, remain unclear. So that, further analysis of identified bands 700 bp which was estimated as a part of *phaC* (1796 bp) is required, such as DNA sequencing of PCR products by using *phaC* primer and southern blot analysis in order to confirm the presence of *phaC* gene sequence.

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Authors' contributions

EM, AP, CN designed the study. CN carried out the laboratory work. CN, EM, AP analyzed the data. CN, EM wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare no competing interest.

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