Menara Perkebunan 87(2), 123-130
p-ISSN: 0125-9318/ e-ISSN: 1858-3768
DOI: http://dx.doi.org/10.22302/iribb.jur.mp.v87i2.336
Accreditation Number: 21/E/KPT/2018

Regeneration of P5CS-transformed oil palm plantlets mediated by *Agrobacterium tumefaciens*

Regenerasi planlet kelapa sawit hasil transformasi dengan gen P5CS melalui Agrobacterium tumefaciens

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Diterima tgl 27 Mei 2019 / disetujui tgl 6 Oktober 2019

Abstrak

Cekaman kekeringan dapat mempengaruhi produktivitas tanaman perkebunan. Rekayasa genetika merupakan salah satu cara untuk meningkatkan produktivitas tanaman perkebunan penting seperti kelapa sawit. Tujuan dari penelitian ini adalah melakukan perekayasaan kelapa sawit melalui introduksi gen P5CS dengan transformasi berbasis Agrobacterium *untuk meningkatkan ketahanan tanaman terhadap cekaman kekeringan. Pada penelitian ini perakitan kelapa sawit transgenik yang tahan terhadap cekaman kekeringan dilakukan melalui transformasi gen P5CS (Δ[1]-pyrroline-5-carboxylate synthetase) ke dalam kalus embriogenik (*embryogenic calli – *EC) menggunakan* Agrobacterium*. Plasmid pBI_P5CS yang membawa gen P5CS ditransfer dari* Escherichia coli *XL1 Blue ke* Agrobacterium tumefaciens *AGL1 melalui konjugasi. Selanjutnya klon* Agrobacterium *yang membawa plasmid pBI_P5CS digunakan untuk menginfeksi kalus embriogenik kelapa sawit dengan perlakuan 100 ppm asetosiringon. Kalus transforman diregenerasi pada media de Fossard (DF) yang ditambahkan 50 ppm kanamisin dan 250 ppm sefotaksim. Kalus transforman diseleksi melalui uji GUS dan metode PCR menggunakan primer NPTII dan P5CS1. Uji GUS dilakukan untuk menyeleksi kalus transforman yang ditunjukkan dengan reaksi positif pembentukan warna biru pada kalus yang berhasil ditransformasi dengan konstruk pBI_P5CS. Pengujian dengan menggunakan PCR memberikan hasil positif dengan adanya profil pita PCR pada visualisasi menggunakan pewarnaan SYBR Green, yang menunjukkan amplikon berukuran ~ 0,7 kb untuk gen NPTII dan ~ 0,4 kb untuk gen P5CS pada elektroforesis dengan gel agarosa. Hasil dari penelitian ini adalah diperolehnya kalus transforman terseleksi yang telah diregenerasi dan tumbuh menjadi planlet.*

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[Kata kunci: cekaman kekeringan, Elaeis guineensis *Jacq., rekayasa genetika, planlet]*

Abstract

Environmental abiotic stressors particularly drought has detrimental effects upon the productivity of estate crops. Increasing the crop tolerance towards drought stress through genetic engineering is one of the strategies employed to maintain steady productivity of valuable crop, i.e. oil palm. The aim of this study was to engineer oil palm with a better tolerance towards drought by introducing *P5CS* (Δ[1]-pyrroline-5-carboxylate synthetase) gene via *Agrobacterium*–mediated transformation into embryogenic calli (EC). The pBI_P5CS plasmid harboring P5CS gene was transferred from *Escherichia coli* XL1 Blue to *Agrobacterium tumefaciens* AGL1 by conjugation. The positive clone of transformed *Agrobacterium* was then used to infect oil palm EC by the addition of 100 ppm acetosyringone. The transformed ECs were regenerated in the de Fossard (DF) media supplemented by 50 ppm kanamycin and 250 ppm cefotaxime followed by GUS assay and PCR-based screening using NPTII and P5CS1 primers. The positive EC clones were confirmed by GUS assay, which produced blue coloration on positive transformed oil palm EC. A positive result of PCR screenings was depicted by PCR products in SYBR Green staining gel agarose electrophoresis with the expected band size of ~ 0.7 kb for the NPTII gene and ~ 0.4 kb for the P5CS gene. This study has successfully selected and regenerated pBI_P5CS transformed oil palm embryogenic calli into plantlets.

[Keywords: drought tolerance, *Elaeis guineensis* Jacq., genetic engineering, plantlets]

Introduction

Abiotic factors can influence plant growth and productivity (Pandey *et al.*, 2017). Environmental stressor particularly drought has detrimental effects upon the productivity of valuable crops, e.g.

oil palm (Oettli *et al.*, 2018; Syarovy *et al.*, 2018), rubber tree (Lestari *et al.*, 2017), and sugarcane (Azevedo *et al.*, 2011). The climate fluctuation affects the productivity of oil palm by changing the average annual temperature and rainfall rate. La Niña caused severe drought in Malaysia, which had the highest reduced oil palm yield in 2002 (Dislich *et al.*, 2017). Moreover, it has been projected that the oil palm productivity in Malaysia will be decreased to 30% if the average temperature increases by 2^{o}C and the rainfall decrease by 10% (Paterson *et al.*, 2017).

During the long dry season, the plant responds to the environmental cue, i.e. low air moisture, by closing the stomata to reduce the loss of water (Urban *et al.*, 2017). However, the closure of stomata diminishes the carbon dioxide intake by the leaves, thus reducing the overall photosynthetic rate and yield (Haworth *et al.*, 2016). Furthermore, the closed stomata prevent the molecular oxygen from diffusing out to the atmosphere, causing photorespiration (Eisenhut *et al.*, 2017). It can further lower the photosynthetic output by producing 2-phosphoglycolate acids, which cannot be anabolized through the Calvin-Benson cycle (Flügel *et al.*, 2017).

The drought stress can be very detrimental to oil palm, which requires daily watering in the amount of 4-5 mm and 1000-2000 mm annually (Jazayeri *et al.*, 2015). There is about a ten percent loss in oil palm productivity for every 100 mm of water deficit annually (Carr, 2011). The drought stress affects not only the productivity of oil palm by reducing the photosynthetic yield but also decrease the number of male and female inflorescence thus decreasing the number of potential productive fruit bunch (Corley & Tinker, 2015). Currently, it is very difficult to arrange for irrigation in oil palm plantation to alleviate drought stress during the long dry season, due to economic, technical, and agricultural reasons. Therefore, it is important to seek other means to maintain steady oil palm productivity during climate fluctuation and drought.

High tolerance plant to drought stress can be developed through genetic engineering. It can be conducted by introducing the *P5CS* gene, which encodes for Δ^1-pyrroline-5-carboxylate synthetase (Turchetto-Zolet *et al.*, 2009). The P5CS is an enzyme involved in the biosynthesis of proline, an amino acid which has a crucial role in plant response to drought stress (Wei *et al.*, 2016). Proline acts as osmolytes, antioxidant, metal chelator, and signalling molecule which is implicated in the maintenance of cell turgor, membrane stabilization during drought stress, and reducing reactive oxygen species (ROS) (Ben Rejeb *et al.*, 2014). The overexpression of P5CS has proven to increase drought tolerance in sugarcane (Li *et al.*, 2018).

Here, this research was aimed to develop drought tolerant oil palm by introducing *P5CS* gene into embryogenic calli using *Agrobacterium*-mediated transformation. The transformed calli were regenerated and screened using GUS assay and PCR-based selection.

Materials and Methods

Transfer of pBI_P5CS constructs from Escherichia coli *XL1 Blue to* Agrobacterium tumefaciens *AGL1*.

The pBI_P5CS construct was built using the *P5CS* gene insert from *Vigna aconitifolia*, kindly provided from Dr. Desh P Verma, Ohio State University (Minarsih *et al.*, 2001; Minarsih *et al.*, 2015). It was transferred from *E. coli* XL-1 Blue to *A. tumefaciens* AGL1 by conjugation. The transformed *A. tumefaciens* were screened for positive clones using supplementation of 50 ppm of kanamycin as selectable antibiotic resistance.

Agrobacterium-*mediated transformation of oil palm embryogenic calli (EC) and regeneration of transformed EC into plantlets*.

The transformation followed a method by Izawati *et al.* (2012) with some modifications. Briefly, the oil palm (Tenera) embryogenic calli (EC) were retrieved from the explants treated by temporary immersion system (TIS) (Marbun *et al.*, 2015).

The EC were pierced with a sterile toothpick and co-cultured with the positive *A. tumefaciens* clone in growth media supplemented with 100 ppm acetosyringone in a dark condition for 48 hours. The transformed EC were then grown in the de Fossard (DF) (Sumaryono & Riyadi, 2011; Sinta *et al.,* 2011) media supplemented with 50 ppm kanamycin and 250 ppm of cefotaxime. The positive clones of oil palm EC were sub-cultured in fresh DF media every month to regenerate the EC to plantlets.

Transformed calli screening using GUS assay and PCR-based method.

The GUS assay was conducted using standard protocol (Jefferson, 1987) with minor modifications. Briefly, the calli were fixated using 4% ice-cold formaldehyde for 30 minutes, followed by washing using an ice-cold buffer for up to 60 minutes. The X-Gluc substrate was infiltrated into the calli assisted by agitation at 37^{o}C. The calli-containing infiltration solution then was incubated overnight in the dark condition. The calli were rinsed using sterile water, and the chlorophyll contents were removed using 70% ethanol. The screening for positive oil palm EC clones harbouring pBI_P5CS was also done by PCR-based selection using NPTII and P5CS1 primers.

The first primer pairs targeted the kanamycin selectable marker while the second ones targeted the P5CS insert gene. Successful PCR reaction yielded ~ 0.7 kb products from the first primer and ~ 0.4 kb from the second primer. The amplifications were programmed to run for 35

cycles for *NPTII* amplification as follow: pre-denaturation at 94°C for a minute, denaturation at 94°C for a minute, annealing at 58°C for a minute, extension at 72°C for 3 minutes and final extension at 72°C for 7 minutes. Meanwhile, the cycles for *P5CS* amplification as follow: pre-denaturation at 94°C for 30 seconds, denaturation at 94°C for 45 seconds, annealing at 55°C for 45 seconds, extension at 72°C for 2 minutes and final extension at 72°C for 7 minutes. The PCR products were visualized on 0.8% agarose gel electrophoresis stained by SYBR green (peqGREEN). All oligonucleotides used in this study were listed in Table 1.

Results and Discussion

The screenings of transformed oil palm embryogenic calli (EC) using GUS assay and PCR-based method

The *Agrobacterium*-mediated transformation of oil palm embryogenic calli (EC) was carried out by adding 100 ppm acetosyringone to promote the transfer of Ti-based pBI_P5CS construct (Figure 1) into the targeted calli. The transformed EC were screened for positive clones using selective media containing 50 ppm kanamycin and 250 ppm cefotaxime. The kanamycin was used as the selectable marker in the pBI_P5CS plasmid construct while cefotaxime was used to eradicate excess *Agrobacterium* growth. The transformed oil palm EC was expected to grow in the antibiotic-supplemented media because it acquired antibiotic resistance after a successful transformation. This screening is a common protocol in retrieving positive EC clones which can grow under the influence of kanamycin.

The screening of transformed oil palm embryogenic calli was firstly assayed using GUS. The principle of GUS assay is the formation of 5-bromo- 4- chloro- 3-indoyl product from 5-bromo-4- chloro- 3- indoyl- β- D- glucoside (X-Gluc) substrate. The product was catalysed by β-glucoronidase (GUS) enzyme introduced after oil palm EC *Agrobacterium*-mediated transformation by pBI_P5CS construct. The 5-bromo-4-chloro-3-indoyl product was then oxidized to form blue coloured indigo insoluble compounds under the influences of ferro- and ferricyanide reagents (Lee & Schöffl, 1997).

The transformed oil palm EC, which harboured pBI_P5CS containing GUS reporter after successful transformation, was shown to produce blue-stained EC, as shown in Figure 2. The overnight immersion (Figure 1a) was noted to be the optimal condition in this research (Figure 2a). This result was supported by other studies, which showed overnight immersion in room temperature as the optimal condition (Lee & Schöffl, 1997). The prolonged incubation until three days did not enhance the colourisation (Figure 2b). Here, the GUS assay was also proven to be robust to select for positive EC clones since non-transformed EC would not yield blue-stained EC even after prolonged incubation (Figure 2c).

Table 1. Oligonucleotides used in this study
Tabel 1. Oligonukleotida yang dipakai pada penelitian ini

No. *No.*	Primer identity *Identitas primer*	Sequences *Sekuens*	Amplicon Sizes *Ukuran amplikon*
1.	P5CS1 F	CATGGAGAGCGCGGTGGATC	~ 0. 4 kb
2.	P5CS1 R	CTTCACAGTCTCAGTAAGCTGC	
3.	NPTII F	GAGGCTATTCGGCTATGACT	~ 0.7 kb
4.	NPTII R	ATCGGCAGCGGCGATACCGT	

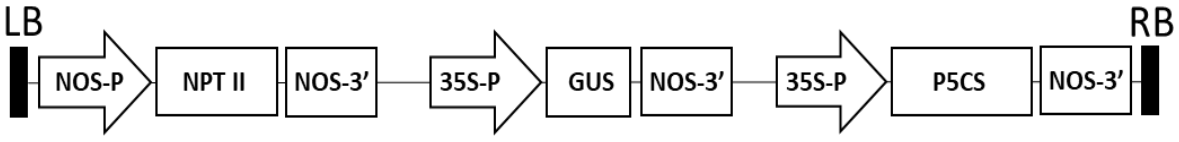

Figure 1. Illustrated map of construct containing kanamycin resistance (*NPTII*), *GUS* (β-Glucoronidase), and *P5CS* genes (Adapted from Hong *et al.,* 2000)

Gambar 1. Ilustrasi peta konstruk yang mengandung gen resistensi terhadap kanamisin (NPTII), GUS (β-Glukoronidase), dan P5CS (Diadaptasi dari Hong *et al.,* 2000)

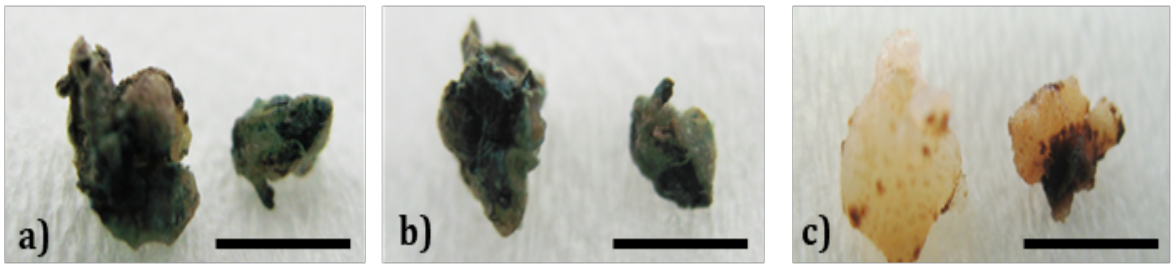

Figure 2. Oil palm embryogenic calli (EC) after various period of immersion in GUS-staining solutions: a) calli after overnight immersion, b) calli after three days of immersion, and c) negative controls (non-transformed calli) with no apparent bluish colour even after three days of immersion. *Bars*, 0.3 cm

Gambar 2. Kalus embriogenik kelapa sawit setelah perlakuan perendaman dengan reagen GUS: a) kalus setelah perendaman semalam, b) kalus setelah perendaman selama tiga hari, dan c) kontrol negatif (kalus non-transforman) yang tidak berwarna biru setelah tiga hari perendaman. Garis, 0.3 cm

The positive EC clones were further verified by PCR-based selection targeting NPTII selectable gene and P5CS transgene. The size of targeted PCR products from *nptII* and *P5CS* genes in pBI_P5CS constructs were ~ 0.7 kb and 0.4 kb, respectively. The results were depicted in Figure 3. The results of PCR-based screenings transformed oil palm EC using NPTII primers were successful as the target PCR product with the size of ~ 0.7 kb appeared on some of the EC samples (Figure 3a). Further PCR confirmation using NPTII primers on embryo and shoot samples were also conducted, yielding some visible ~ 0.7 kb PCR products (Figure 3b). The visualisations of PCR products amplified from embryos and shoots clearly showed degrees of stability of transgenes as the calli grew through the regeneration phases. The thicknesses of PCR bands showed a copy number of amplicons which gradually became thinner on several lanes. The gradual decreased of amplicon copy numbers is correlated to the efficiency of *Agrobacterium*-mediated transformation; lower efficiency of transformation will yield thinner amplicon thickness. It also correlated to the stability of transgenes since the same exact copy number of transgenes is not always distributed evenly in the daughter cells of actively dividing calli. Therefore, transformed calli showing only consistent PCR band, e.g. calli with PCR band from lane three which later grown into shoots from lane 6, were picked and monitored for regeneration to the later phases. The case of decreased intensity of PCR amplicons is also commonly happened in the generation of transgenic *Citrus*. It was caused by the higher protection of non-transformant cells by the surrounding transformed cells and also persistency of kanamycin-resistant *Agrobacterium* after long period of co-cultivation (Dominguez *et al.*, 2004).

The PCR-based confirmation was further done using P5CS1 primers used in the previous research which targeting the *P5CS* inserted gene (Fitranty et al, 2003). Figure 4 depicted the result of the PCR validation on oil palm embryogenic calli (EC). Whilst the PCR on embryo, shoot, and plantletshowed several nonspecific bands appeared after amplification using P5CS1 primers, beside the specific bands showing P5CS transgene (the ~ 0. 4 kb band) among transformed samples (data not shown). Nonspecific bands were also visible after PCR amplification, demonstrating that the primers can also amplify nonspecific targets in the oil palm genome. However, these specific bands were re-confirmed after successful amplification in the control of positive samples using pBI_P5CS plasmid while it did not exist in the negative control samples using distilled water as a template. The result also will be further confirmed by sequencing of the PCR amplicons of the P5CS gene. The PCR-based screenings were able to demonstrate the success of *Agrobacterium*-mediated transformation of oil palm by the screening of embryogenic calli harbouring pBI_P5CS construct which included antibiotic selectable marker *nptII* genes and *P5CS* gene insert.

Regeneration of transformed oil palm embryogenic calli (EC) into plantlets

The transformed oil palm EC clones were regenerated into plantlets by series of sub-cultures in de Fossard (DF) media supplemented with 50 ppm of kanamycin (Figure 5). The non-transformed EC (Figure 5a) were failed to grow and differentiate into embryos inversely to the successful growth of the transformed EC (Figure 5b). The supplementation of kanamycin inhibits protein synthesis in mitochondria and chloroplasts, thus preventing the growth of non-transformed EC. It is caused by the binding of kanamycin to the 30S of ribosomal RNA residing in plant mitochondria and chloroplasts which play an essential role in assisting mRNA binding to the small subunit of the ribosome (Conte *et al.*, 2009).

The ability of transformed oil palm EC to grow under the influence of kanamycin came from the expression of the *nptII* gene in the pBI_P5CS construct. It encodes the neomycin phosphotransferase, which exports the kanamycin out of the cell (Carrer *et al.*, 1993). Therefore, it confers the resistance ability towards the kanamycin. This resistance enabled the growth of transformed oil palm EC, which later developed into embryos (Figure 5c) after two months incuba-

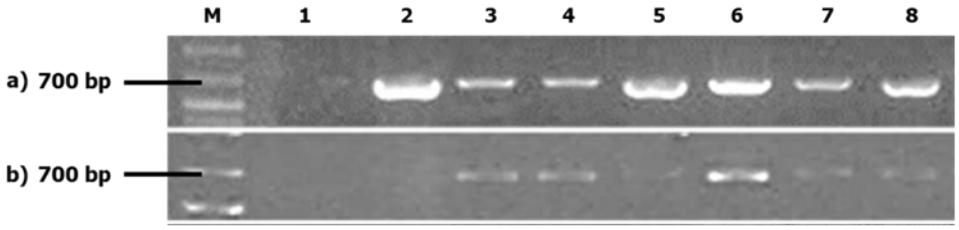

Figure 3. Validation of calli transformation for oil palm. *a)* PCR using NPTII primers on oil palm embryogenic calli (EC) (M: 1kb plus DNA marker; 1: negative control (H_2O); 2: positive control (pBI_P5CS plasmid); 3–8: transformed calli), *b)* PCR using NPTII primers on oil palm embryo and shoots (M: 1 kb plus DNA marker; 1: negative control (H_2O); 2: non-transformed embryo; 3–5: transformed embryos; 6–8: transformed shoots)

Gambar 3. Validasi transformasi pada kelapa sawit. a) *PCR dengan primer NPTII pada kalus embriogenik (M: Marka DNA 1kb plus; 1: kontrol negatif (H_2O); 2: kontrol positif (plasmid pBI_P5CS); 3–8: kalus transforman),* b) *PCR dengan primer NPTII pada embrio dan tunas kelapa sawit (M: Marka DNA 1kb plus; 1: kontrol negatif (H_2O); 2: embrio non-transforman; 3–5: embrio transforman; 6–8: tunas transforman)*

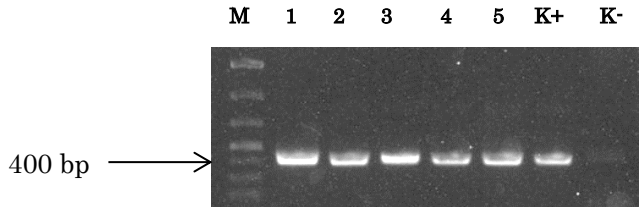

Figure 4. PCR using P5CS1 primers on embryogenic calli (M: 1kb plus DNA marker; 1-5: transformed embryogenic calli; K+: positive control (pBI_P5CS plasmid);K-: negative control (H_2O)

Gambar 4. PCR dengan primer P5CS1 pada kalus embriogenik (M: 1kb plus DNA marker; 1-5: kalus embryogenik transforman; K+: kontrol positif (plasmid pBI_P5CS); K-: kontrol negatif (H_2O;

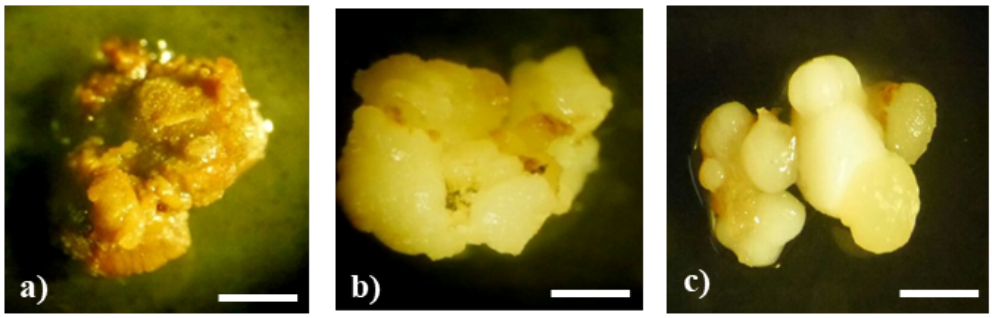

Figure 5. The growth of pBI_P5CS-transformed oil palm embryogenic calli/EC into initial shoots. *a)* negative control (non-transformed EC), *b)* transformed EC, and *c)* transformed somatic embryos. *Bars*, 0.3 cm

Gambar 5. Pertumbuhan kalus embriogenik kelapa sawit menjadi tunas inisial. a) *kontrol negatif (kalus embriogenik non-transforman),* b) *kalus embriogenik transforman, dan* c) *embrio somatik transforman. Garis, 0.3 cm*

tion of post-transformation. This resistance enabled the growth of transformed oil palm EC, which later developed into embryos (Figure 5c) after two months incubation. Altogether, the production of viable plantlets from EC was achieved in two years (Figure 6). The plantlets are now ready for subsequent characterization in pre-nursery settings.

The transgenic oil palm plantlets harbouring pBI_P5CS are ready for further downstream characterization, i.e. drought stress assay. Such assay can be conducted *in vitro* easily using desiccation exposure mimicked by PEG (polyethylene glycol) or gradient of sodium chloride added to the media. Improved tolerance towards desiccating/dehydrating agent is highly correlated to the respective improved traits. Therefore, the *in vitro* validation should be conducted carefully, also ensuring a successful field trial.

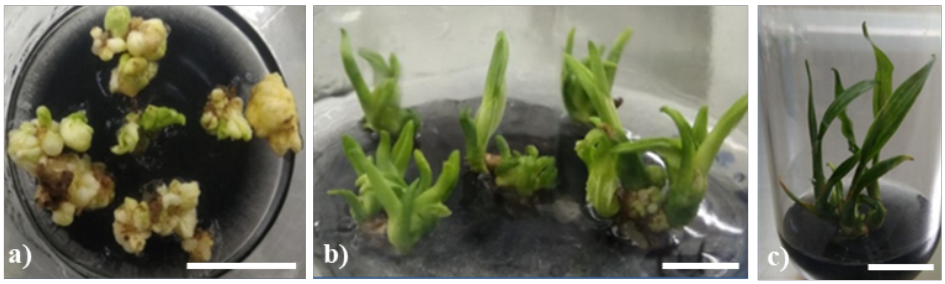

Figure 6. The growth of pBI_P5CS-transformed oil palm embryos into plantlets. *a*) somatic embryos with initial shoots (± 6 months old), *b*) shoots (± a year old), and *c*) plantlets (± two years old). *Bars*, 1 cm (*a-b*), 2 cm (*c*)

Gambar 6. Pertumbuhan embrio transforman pBI_P5CS menjadi planlet. a) *embrio somatis dengan tunas inisial* (umur 6 bulan)*,* b) *tunas* (umur satu tahun)*, dan* c) *planlet* (umur dua tahun)*. Bar, 1 cm (*a-b*), 2 cm (*c*)*

According to several studies, proline accumulation has been correlated to the improvement of drought tolerance in plants (Hayat *et al.*, 2012; Kavi-Kishor & Sreenivasulu, 2014; Amini *et al.*, 2015). Proline has a vital role in drought tolerance through the modulation of pathways, including salicylic acid (SA)-induced signalling. In wheat cultivars, the application of SA and silicone induced the expression of P5CS gene, which then followed by the proline accumulation inhibiting electrolyte leakage during drought stress (Maghsoudi *et al.*, 2018). It can also capture radical oxygen species (ROS), which is elevated in plants under drought stress (Noctor *et al.*, 2014). The ROS accumulation is in conjunction with the photorespiration which occurs during the stomatal closure after drought stress (Voss *et al.*, 2013).

During the stomatal closure, the low photosynthetic yield can be amended by proline due to its conversion to glutamate in mitochondria (Zhang & Becker, 2015). This conversion yields FADH2 and NAD(P)H, which then can be fed into the Calvin-Benson cycle (Wu *et al.*, 2017). Furthermore, the degradation of proline can also supply nitrogen as a nutrient which account for the increased productivity recovery after drought stress in tolerant plants (Albert *et al*., 2012).

Altogether, overexpression of P5CS in sugarcane (Li *et al.*, 2018), wheat (Maghsoudi *et al.*, 2018), and soybean (Soleimani *et al.*, 2015) can improve plants tolerance to drought stress. Therefore, the P5CS transgene expression in oil palm could potentially improve the plant drought tolerance maintaining high productivity as has successfully done in other crops.

Conclusions

The introduction of *P5CS* gene into oil palm embryogenic calli (EC) was successfully conducted using *Agrobacterium*-mediated transformation which opens a possibility of developing drought tolerance oil palm. The positive oil palm EC clones were successfully verified using GUS assay and PCR-based method targeting *nptII* selectable marker and *P5CS* insert. The transgenic oil palm was also successfully regenerated into plantlet which is ready for further characterization.

Acknowledgements

The authors thanked Elizabeth Monica Sitorus, Nazhirotul Ilmiyah, and Marini for the assistance during the lab works. These works were partly funded by BPDPKS (*Badan Pengelola Dana Penelitian Kelapa Sawit – Republik Indonesia*).

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