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OPTIMIZATION OF *Agrobacterium*-MEDIATED GENETIC TRANSFORMATION OF *oshox4* GENE IN SATOIMO TARO (*Colocasia esculenta* var. antiquorum)

Optimasi Transformasi Genetik Gen *oshox4* Melalui *Agrobacterium* Pada Talas Satoimo (*Colocasia esculenta* var. antiquorum)

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ABSTRACT
*Satoimo (*Colocasia esculenta *var. antiquorum) can be considered as an alternative food to support food diversification. The aim of this research was to obtain an optimum condition of the genetic transformation method of Satoimo taro through* Agrobacterium*-mediated genetic transformation using binary vector harboring gene construct of oshox4 and hpt gene as a selectable marker to produce transgenic plants. Plant materials that were used in this study were meristem, root, leaf, petiole, and basal stem. Transformation procedures were carried out using three different co-cultivation periods (1, 2, and 3 days) and different optical densities of* A. tumefaciens *cells (0,5 and 0,7). Furthermore, callus induction was performed on MS medium containing 2.4-D, cefotaxime, hygromycin and then regenerated on MS medium containing thidiazuron and hygromycin. The putative transgenic plantlets were selected on MS medium containing hygromycin and analyzed using PCR. The results showed that putative transgenic plants derived from the basal stem could survive on a selection medium containing hygromycin and based on PCR analysis some of them contained the oshox4 and hpt genes.*

Keywords: Colocasia esculenta *var. antiquorum, drought, hpt gene, oshox4 gene, stress*

ABSTRAK
Satoimo (*Colocasia esculenta* var. antiquorum) dapat dijadikan sebagai alternatif pangan untuk mendukung penganekaragaman pangan. Tujuan penelitian ini adalah mendapatkan kondisi optimal untuk transformasi genetik talas Satoimo menggunakan gen oshox4 yang dimediasi oleh *Agrobacterium tumefaciens*. Transformasi genetik dilakukan dengan menggunakan vektor biner yang mengandung gen oshox4 dan gen hpt. Bahan tanaman yang digunakan dalam penelitian ini adalah meristem, akar, daun, tangkai daun dan pangkal batang talas Satoimo. Transformasi dilakukan dengan menggunakan tiga perlakuan waktu ko-kultivasi (1, 2, dan 3 hari) serta dua optical density (0,5 dan 0,7). Selanjutnya dilakukan induksi kalus pada media MS yang mengandung 2.4-D, cefotaksim, hogromisin dan diregenerasikan pada media MS yang mengandung thidiazuron dan higromisin. Plantlet putatif transgenik diseleksi menggunakan media MS yang mengandung higromisin serta dianalisis menggunakan PCR. Hasil penelitian menunjukkan bahwa tanaman putatif transgenik yang berasal dari pangkal batang dapat bertahan hidup pada media seleksi yang mengandung higromisin dan berdasarkan analisis PCR, beberapa di antaranya mengandung gen oshox4 dan gen hpt.

Kata Kunci: cekaman, *Colocasia esculenta* var. antiquorum, gen hpt, gen oshox4, kekeringan

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INTRODUCTION

Food demand has been increasing by year. The average of daily calories consumption in Indonesia increased from 1 859.3 kcal (2014) to 1 992.69 kcal 92015) (BPS 2016). Indonesia's rice import in 2014 until 2015 increased from 844,163.7 tons to 861,601 tons (BPS 2016), this condition occurs due to conversion of rice field into non-agricultural uses. Another problem is massive decrease of agricultural land area in the form of rice field conversion.

Based on the processing of high-resolution spatial data in various regions of Indonesia, it is estimated that rice fields, which in 2015 were 8.1 million ha, are predicted to reduce only about 5.1 million ha in 2045 (Mulyani et al. 2016). Therefore, it is needed to increase food production, including agricultural extensification using dry land and food diversification to reach the national food sufficiency target. The application of conservation agriculture principles to dry climate dry land has been successfully carried out for corn commodities in the provinces of West Nusa Tenggara and East Nusa Tenggara (Mulyani and Mamat 2019).

One of the main alternative staple foods which can substitute rice is tuberous plants. One type of tuber that is a popular carbohydrate source in Indonesia is taro. One type of taro that has the potential to be developed is Satoimo taro (*Colocasia esculenta* var. antiquorum). Taro tubers contain carbohydrates, protein, and fat. Compared to other types of carbohydrates, such as potatoes, rice, and wheat, Satoimo taro possess a comparative nutritional content in terms of calories, protein, carbohydrates, and calcium (Novita et al. 2017).

Another benefit of Saoimo taro is its low-fat content (0.17–0.2%), and fiber content of 5.3–16.18 g (Novita et al. 2017) which is higher when compared to the fiber content of cassava tubers (0.1–3.7 g) (Zekarias 2019) and potato (1–3 g) (Beals, 2019). The benefit of Satoimo taro nutrient content for health is hyaluronic acid, a substance that is naturally produced in the tuber to produce collagen (ITPC 2014) and glucomannan (Maretta et al. 2020). Satoimo taro cultivation requires rainfall of 1,000 mm/year, and an optimal temperature of 21 - 27 ºC with 50% humidity (BPTP Bengkulu 2015). The potential land for planting area for this plant is dry land due. The availability of dry land in Indonesia for agricultural development is 24.79 million ha (Ritung et al. 2015). This is a great resource for increasing food production.

Increasing the production of agricultural crops in a dry land, superior plants that are tolerant to abiotic stresses, especially drought stress are needed. One method to obtain superior seeds is genetic transformation techniques using genes for drought resistance. The principal procedure of genetic transformation is the insertion of a gene target into the T-DNA plasmid of *Agrobacterium tumefaciens*, then inoculated into plant tissue (Sukamto et al. 2017). The *Agrobacterium*-mediated genetic transformation method has been widely used in many plant species such as rice (Islam et al. 2015, Rahmawati et al. 2019), potato (Bánfalvi et al. 2020), taro (He et al. 2013), soybean (Li et al. 2017), sweet potato (Lan et al. 2018), anthurium (Hosein et al. 2012), yam (Nyaboga et al. 2014), citrus (Ghaderi et al. 2018), cassava (Cauhan et al. 2015, Elegba et al. 2021), horse gram (Amal et al. 2020), *Hevea brasiliensis* (Udayabhanu et al. 2022).

One of the genes that can be used to produce drought tolerant transgenic plants is a group of transcription factors, i.e. the *oshox* gene family which plays an important role in the tolerance mechanism to drought stress. The *oshox4* gene is a member of the HD-ZIP 1 group. The HD-Zip I gene is associated with adaptation to drought stress (Purwantomo 2007). Overexpression of the *oshox4* gene in rice can increase drought resistance (Zhou et al. 2015). O*shox6* gene has been successfully overexpressed in rice (Rahmawati et al. 2019). The genetic transformation of Satoimo taro with the *oshox4* gene through *A. tumefaciens* is expected to produce transgenic plants that are resistant to drought stress.

The objective of this study was to obtain optimum genetic transformation conditions involving explants type, co-cultivation period, and optical density of *A. tumefaciens* that can be used to introduce *oshox4* gene construct into the Satoimo taro genome.

MATERIALS AND METHODS

Location and time

Research was conducted in August 2014 to June 2018 at Laboratory of Molecular Genetics and Biosynthetic Pathway Alteration

– Research Centre for Biotechnology – Indonesian Institute of Sciences (LIPI).

Plant materials

Taro cv Satoimo which is grown at Germplasm Collection Garden, Cibinong Science Center – Botanic Garden was used as plant material.

Optimization of transformation protocol

A. tumefaciens was cultured in Luria-Bertani medium containing kanamycin 50 mg L^{-1} and rifampicin 20 mg L^{-1}) and then diluted into 0,5 and 0,7 optical density of cells. This bacteria carrying the binary vector pCambia 1300-*oshox* that harbor *hpt* gene as a selectable marker linked to *oshox4* gene, both under the control of the constitutive cauliflower mosaic 35S virus (35S CaMV) promoter and terminator (Figure 1).

To establish the *Agrobacerium*-mediated genetic transformation protocol, the following factors which were affected transformation efficiency were evaluated: explants type (meristem, root, leaf, and petiole), cell optical density (OD) (0.5 and 0.7), and cocultivation period (1, 2, and 3 days). Each treatment combination consisted of 50 explants. Explants were dipped in *Agrobacterium* culture for 5 minutes and then co-cultivated on half-strength MS medium for 1, 2, or 3 days. Explants were then transferred to callus induction medium (MS + 2 mg L^{-1} 2,4- D + 150 mg L^{-1} cefotaxime + 10 mg L^{-1} hygromycin) following cultured on regeneration medium (MS + 0,005 mg L^{-1} TDZ + hygromycin), then re-subcultured on MS hormone-free medium containing 10 mg L^{-1} hygromycin. Transformed explants were observed to check the number of explants developed into callus and regenerated into whole plants.

Transformation on the basal stem

The optimum genetic transformation procedure was applied to the basal stem explant. After the transformation process (after co-cultivation), the basal stem of Satoimo taro was sub-cultured in the same medium as the previous experiment. The transformed explants were selected on an MS medium containing hygromycin. The concentration of hygromycin used to select the transformed explants was 10 mg L^{-1} (Salsabila 2015).

Analysis of putative transformants

Genomic DNA was isolated from putative transformant leaves by the method of Edwards et al. (1991) which was modified at the step of lipids and proteins separation using Chloroform: octanol (24:1). The leaves of putative transformants (50 mg) were placed in microtubes and then were ground to be a fine powder with liquid nitrogen. The extraction buffer of 400 µL was added and then incubated at room temperature for 3 min. After incubation, the mixture was centrifugated for 4 min at 12.000 rpm at 4 ^{0}C. The supernatant was collected and then added with 350 µL of chloroform : octanol solution (24:1). The mixtures were then centrifuged at 12.000 rpm for 4 min. The aqueous phase of the mixture was transferred to a new tube and then 300 µL cold isopropanol were added to the tube and left for 15 min at room temperature following centrifugation for 15 min at 12000 rpm. Pellet was washed with a cold 70% ethanol. The pellet was dried and then dissolved with ddH_2O.

The integration test of *oshox4 gene* and *hpt* gene of putative transformants was conducted by PCR (polymerase chain reaction) analysis using a specific sequence primer for *oshox4* and *hpt*. The primer design for *oshox4* was carried out using the Gene Script program with the gene bank accession number AF145728 (Purwantomo 2007). A primer of 5'-GAGGCAGCCCATCGCTCGTC-3' (*forward*) dan 3'-CGCGTCCACGTCGTTCAGCA-5' (*reverse*) were used to amplify of *oshox4* gene. The sequence primer of the *hpt* gene was 5'-GAT GCC GCT CGA AGT AGC G-3 ' and 5'-GCA TCT CCC GCC GCA CGT C-3' (Purwantomo

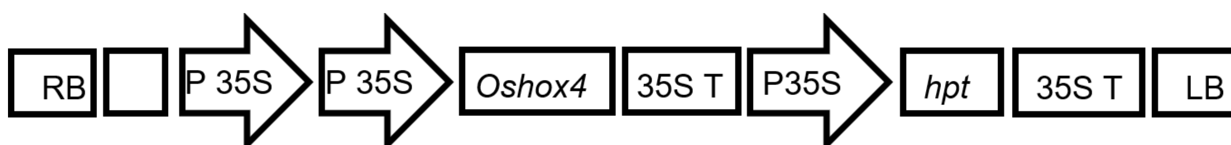

Figure 1. Genetic map of *hpt* and *oshox 4* genes of binary vector (Purwantomo 2007)

2007). PCR reaction mixture was composed of 12,5 µL PCR mix, 1 µL of 50 pmol of both forward and reverse primers, ddH_2O, and 100 ng of DNA template for 25 µL reaction mixture. PCR condition for *hpt* gene were as follow: a denaturing step at 95 ^{o}C for 3 min, followed by 35 cycles at 95 ^{o}C for 30 sec, 58.5 ^{o}C for 45 sec, 72 ^{o}C for 1 minute, and finishing with an extension step at 72 ^{o}C for 10 min. PCR condition for *hpt* gene were as follow: a denaturing step at 95,2 ^{o}C for 5 min, followed by 35 cycles at 95 ^{o}C for 45 sec, 54 ^{o}C for 1 min, 72 ^{o}C for 2 minutes, and finishing with an extension step at 72 ^{o}C for 7 min. PCR products were separated on an 1% agarose and visualized using UV transillumminator after soaked in ethidium bromide solution.

RESULTS AND DISCUSSION

Optimization of genetic transformation protocol

Growth regulators supplementation to MS media in the form of auxin (2,4-D and TDZ, expected to induce somatic embryogenesis and regeneration. The use of 2,4-D and TDZ to induce callus proliferation and shoot regeneration has been widely applied in many plant species involving potatoes (Kamrani et al. 2015), Satoimo taro (Fitriani et al. 2016), and *Myrmecodia tuberosa* (Sari et al. 2018). The selection method using antibiotics is the initial method that can be used to identify putative transgenic plants carrying the *hpt* gene which will produce the enzyme hygromycin phosphotransferase that

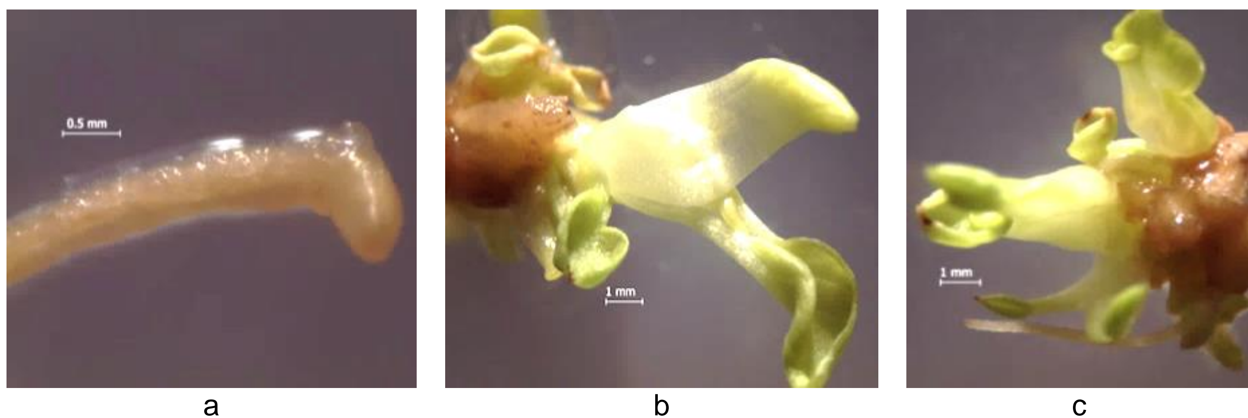

Figure 2. Development of transformed explants with different co-cultivation period.(a) callus derived from petiole with OD 0.5 and co-cultivation of 2 days, (b) callus derived from root with OD 0.5 and co-cultivation period 3 days, (c) callus derived from meristem with OD 0.5 and co-cultivation of 3 days

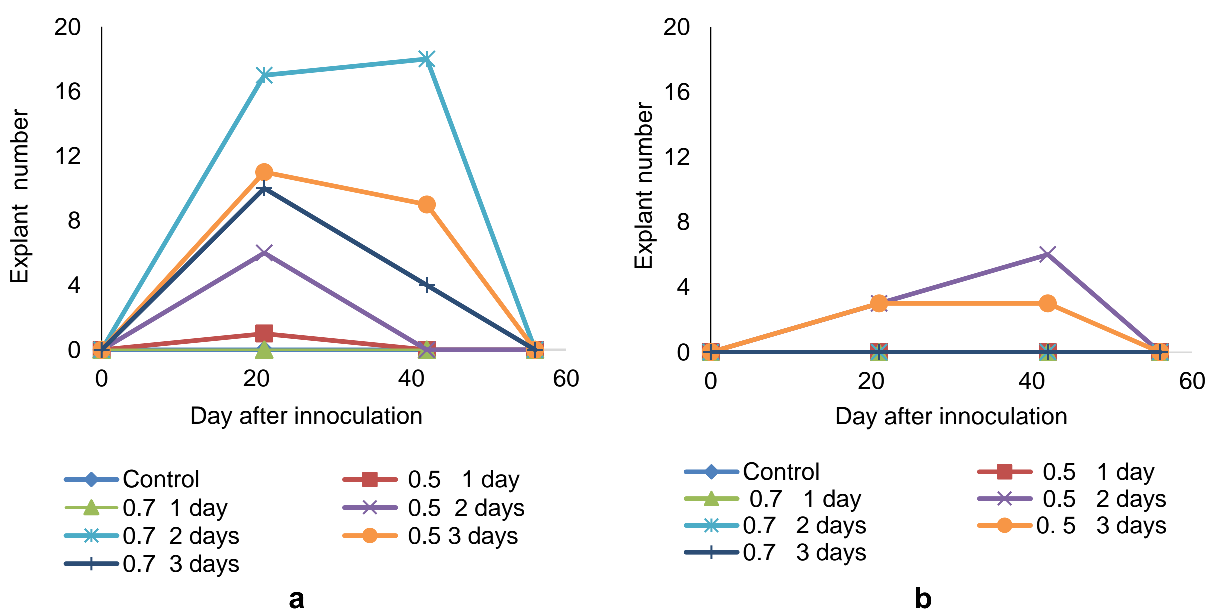

Figure 3. The increasing number of transformed explants of meristem (a) and petiole (b) indicating organogenesis development

phosphorylates hygromycin so that it is not toxic to plants (Holme et al. 2008).

Based on observations at 14 days after inoculation (DAI), it was known that explants producing callus were shoot, petiole, and root meristem, while explants that formed leaves through organogenesis were meristem and petiole (Figure 2). Explants could survive up to 30 DAI that was indicated by the growth of callus and leaves. However, after 30 DAI some of the explants started browning and at 60 DAI all explants died (Figures 3 and 4).

Optical density of *Agrobacterium* solution of optimum genetic transformation are vary among plants, OD 0.9 on rubber trees (Kalawong et al. 2014), OD 0.5 on passion fruit (Asande et al. 2020), and OD 0.6 on *Liriodendron* hybrid (Li et al. 2022). Based on Figures 3 and 4, the OD of 0.5 gives the optimum results, while the best co-cultivation time is 1 (one) day. This result was in line with the research of John et al. (2014) on *Populus deltoides* which showed that the highest transformation efficiency using *A. tumefaciens* was obtained at OD 0.3-0.5 with a co-cultivation time of 1 day, while the type of explant that gave the best results was meristem. Meristem explants can be induced into callus and regenerate into shoots, while the death of explants in this study is due to browning of the tissue (Figure 5). All types of explants (4 types) resulting from the transformation that developed into callus or regenerated into shoots could survive for 30 DAI and then undergo browning of tissue and all died at 60 DAI.

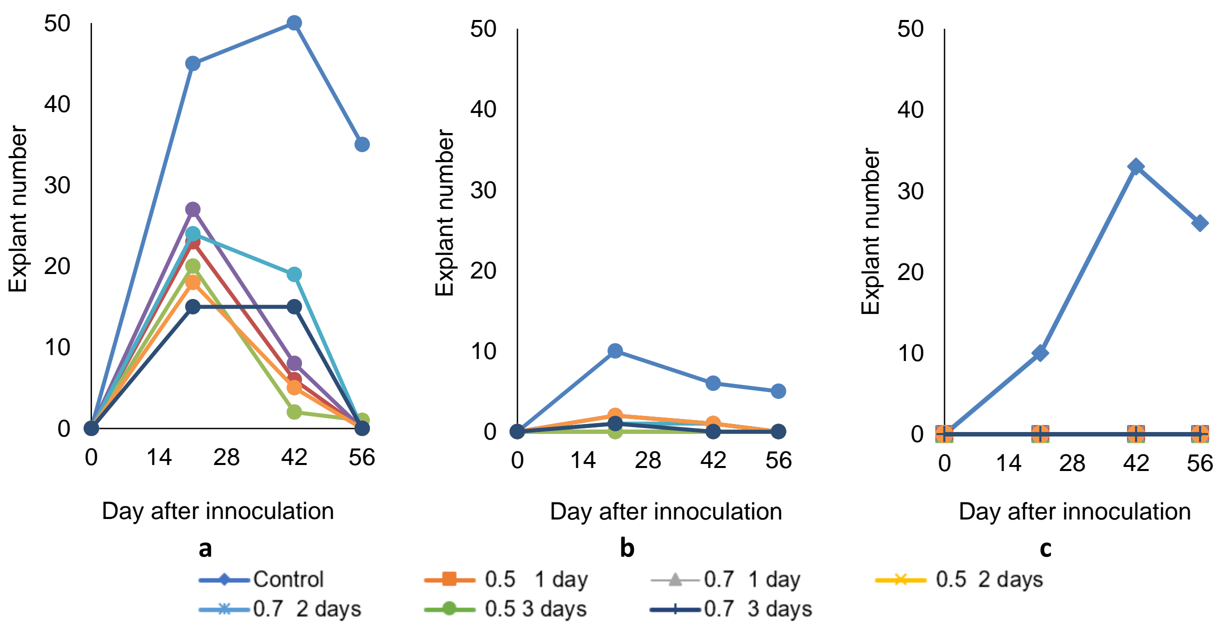

Figure 4. Number of root explant formed callus from meristem (a), petiole (b), and root (c)

Figure 5. The transformed meristem explant that showed browning process

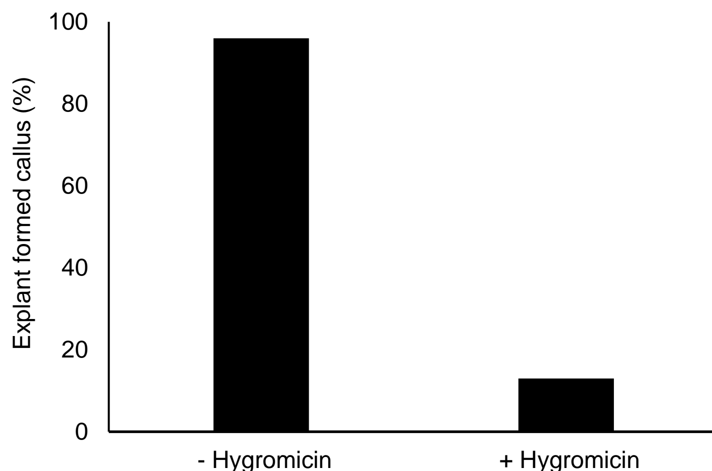

Figure 6. Percentage of stem base explant formed callus

Transformation of the basal stem

The transformation experiment was then repeated using another type of explant, i.e basal stem. The optimum transformation conditions for 4 types of explants (shoot meristem, root, leaf, and petiole) were OD 0.5 and co-cultivation time for 1 day. Observations on the growth of basal stem explants showed that 13% of explants could grow and develop into plantlets on a selection medium containing hygromycin (Figure 6). The callus induction and callus regeneration stages resulting from the transformed stem base explants can be seen in Figure 7.

Confirmation test of the transformants

The base stem explants that survived on the selection medium were then analyzed to test the integration genes of the *oshox4* and *hpt* into the transformed Satoimo taro plant genome. Genomic DNA amplification was carried out using specific *hpt* and *oshox4* primers. The results of amplification of the transformants and control genomic DNA of taro using hpt primers can be seen in Figure 8. Figure 8 shows that among the transformed plants some of them were transgenic plants that contain the *hpt* gene (line 15). The transgenic plants produced the same DNA

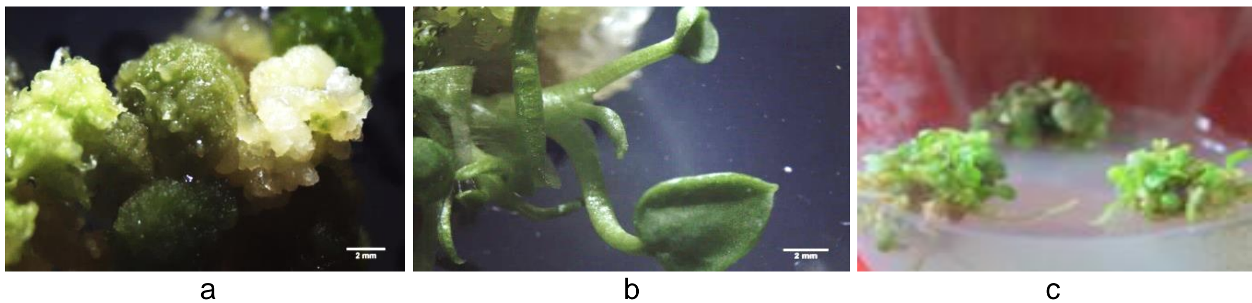

Figure 7. Development stages of transformed stem base Satoimo taro.(a) callus induction, (b) regeneration stage, (c) shoot proliferation and root formation on selection medium containing hygromycin

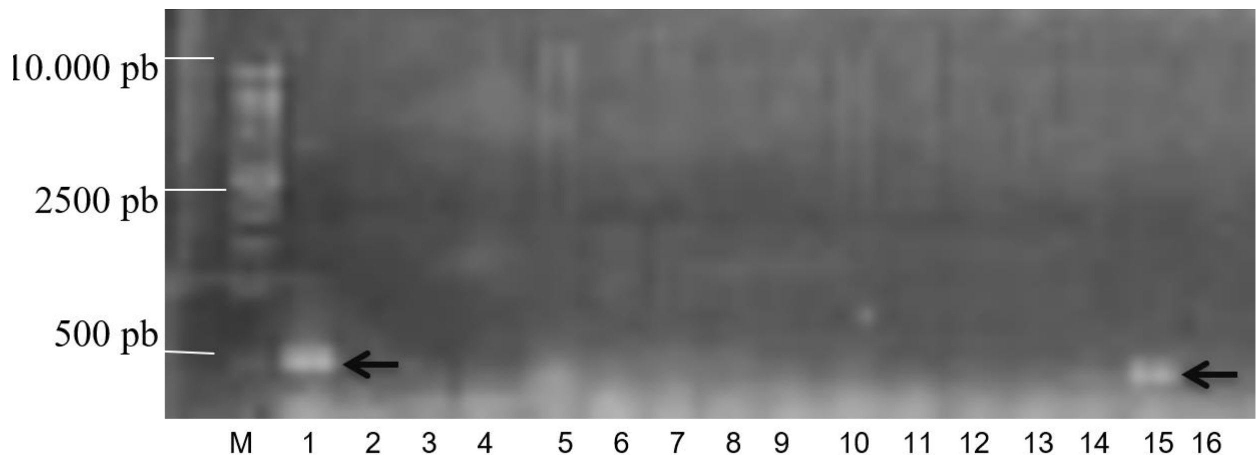

Figure 8. Gene integration test of *hpt*. M: 1 kb DNA *Ladder*, 1: plasmid of pCambia1300-*Oshox4*, 2-3: genomic DNA of wilt type plants, 4-16: transformants plants

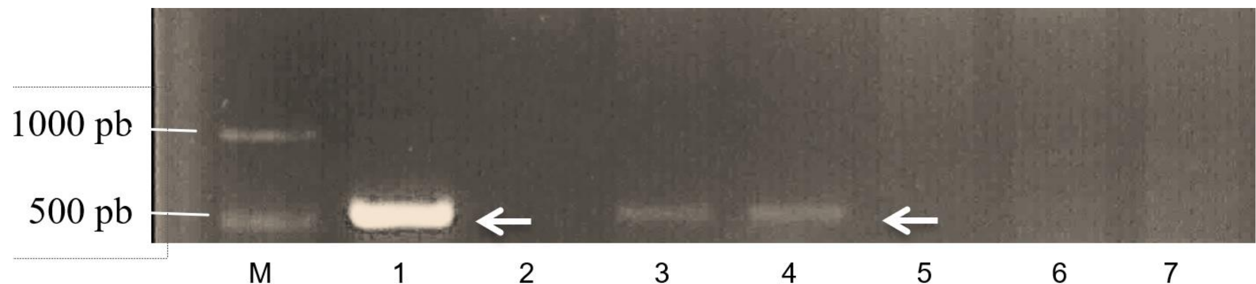

Figure 9. Gene integrstion test of *Oshox4*. M: 1 kb DNA *Ladder*, 1: plasmid of pCambia1300-*Oshox4*, 2: -3: genomic DNA of wilt type plants, 3-7: transformants plants

band of 429 bp in size as the control plasmid (lane 1). This indicates that the *hpt* gene was successfully integrated into the Satoimo taro genome that developed from stem base explants. The *hpt* which encodes the hygromycin phosphotransferase enzyme conferring resistance to hygromycin from *Escherichia coli* is one of the most commonly used antibiotic resistance marker genes for the selection of transformed cells in the development of genetically modified plants (Breyer et al. 2014).

Therefore, these results confirmed that the Satoimo taro plant containing the *hpt* gene is a transgenic plant. The results of the amplification of the *oshox4* gene using the PCR technique can be seen in Figure 9. The *oshox4* gene has been integrated into the transformed Satoimo genome. The sharp band pattern was found in line number 1 while the weak band pattern is found in lines number 3 and 4. The difference in the intensity of the DNA band is probably influenced by the quality and quantity of the DNA template. The poor quality of DNA templates can be caused by contaminants and other metabolites such as phenol protein (Wardani et al. 2017). The *oshox4* gene is a member of the HD-ZIP 1 group. The HD-Zip I gene is associated with adaptation to drought stress (Purwantomo 2007). Furthermore, transgenic Satoimo taro plants containing the *oshox4* gene could respond to drought conditions. The new characteristics of transgenic Satoimo taro, specifically drought resistance, can be evaluated further through a challenge test on media containing osmoregulatory compounds such as polyethylene glycol and sorbitol.

CONCLUSION

In this study, an optimum *Agrobacterium*-mediated transformation system was established in the Satoimo taro using basal stem as an explant. The combination of co-cultivation period for one day and optical density of *A. tumefaciens* 0.5 was the optimum condition to generate transgenic Satoimo taro. This protocol would allow obtaining transgenic taro through the somatic embryogenesis in relatively high transformation efficiency.

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