

Introduction of *Hd3a* gene in IPB CP1 potato cultivar through *Agrobacterium tumefaciens*-mediated transformation under the control of *use 35S CaMV* promoter

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ABSTRACT

IPB CP1 (Chip potato 1) potato cultivar is a new potato varieties obtained by irradiating the Atlantic potatoes at a dose of fifteen gray by the Indonesian National Atomic Energy Agency (BATAN) and researchers at the Bogor Agricultural University. In Indonesia, these potatoes are used as chips. Tuber is a plant organ that serve as storage organ for nutrient and it is used for survival during dry season. One way to increase production of potato is through induction of its tuber. Heading date gene 3a (*Hd3a*) is one of the genes that can regulate flowering time in rice. In addition to inducing flowering, this gene have proven to induce the formation of tuber in *Andigena* potato cultivar. In this study we introduced *Hd3a* gene under the control of *35S CaMV* promoter into the IPB CP 1 potato cultivar to induce formation of its tubers. Genetic transformation was done by co-cultivation method using *Agrobacterium tumefaciens* strain LBA4404 carrying *Hd3a* gene. From the transformation of 157 explants from internodes, we obtained 16.4% and 23.1% as value of transformation efficiency and regeneration efficiency respectively. Six putative transgenic shoots survive on callus induction medium containing 15mg/L of hygromycin antibiotic. Molecular analysis was carried out by using 35S-F primers and Hd-R primers to prove if *Hd3a* gene was integrated into IPB CP 1 potato. PCR analysis showed that all of the six putative transgenic IPB CP 1 potato contained the *Hd3a* gene under the control of *35S CaMV* promoter.

Keywords : *Agrobacterium tumefaciens*, *Hd3a* Gene, IPB CP1

INTRODUCTION

Potatoes (*Solanum tuberosum* L.), the fourth largest global food crop after maize, wheat and rice, is cultivated for its underground storage stems or tubers, which are rich in starch and other nutrients. Due to their high carbohydrate content and relative low farming demands, tuber-bearing species such as potatoes are an important contribution to human dietary needs in many climatic zones, and interest in these staple crops for processed food and other industrial uses is increasing (Wilkinson *et al.*, 1994).

Tuber formation in potatoes (*Solanum tuberosum* L.) is a complex developmental process that requires the interaction of environmental, biochemical and genetic factors. It involves many important biological processes, including formation and growth of stolon, tuber induction, tuber initiation, tuber growth, carbon partitioning, signal transduction and meristem determination (Gregory 1965). Therefore tuber formation is very cardinal focus area for potato breeders and the potato industries.

The mechanism of tuberization has been the subject of considerable investigation by plant scientists and breeders (Notaguchi *et al.*, 2008). These investigations and researches have given rise to several varieties potato cultivar including Nooksack, *Andigena* (*Solanum tuberosum andigena*), Atlantic potatoes, collection of the Bogor Agricultural University (Jala Ipam potato cultivar, IPB CP1 potato cultivar etc.) cultivars. The Atlantic potato cultivars are the best chip potato cultivars in most part of the world especially Asia with its weaknesses

been sensitiveness to bacterial diseases mainly wilt disease (*Ralstonia solanacearum*) and soft rot disease (*Erwinia caratovora*) (Suharsono *et al.*, 2016).

IPB CP1 (*Chip Potato 1*) potato cultivar is a new potato variety obtained by irradiating the Atlantic potatoes cultivar by the Indonesian National Atomic Energy Agency (BATAN) and researchers of the Bogor Agricultural University. IPB CP1 potato cultivar have elongated oval leaves shape, slightly round tuber shape, white bulbs skin color, white tubers, high starch content and low sugar content (Suharsono *et al.*, 2016). Due to its somaclonal variation through radiation, regeneration and resistance selection in vitro, the IPB CP1 cultivar is now resistance to the wilt disease (*Ralstonia solanacearum*) and soft rot disease (*Erwinia caratovora*).

Recent studies have led to the identification of members of the Flowering locus (FT) gene family as major component of the tuber-inducing signal and the characterization of circadian and photoperiodic components involved in the regulation of these genes (Kojima *et al.*, 2002). FT, a well-known floral integrator gene, plays an important role in controlling flowering time in higher plants (Kardailsky *et al.* 1999). More recently, FT and its orthologs have been proposed as florigens, or mobile flowering signals, migrating from leaves to the apical meristem to promote floral initiation (Tamaki *et al.*, 2007). Overexpression of Arabidopsis thaliana FT or ectopic expression of FT orthologs such as Hd3a of *Oryza sativa* L. (Kojima *et al.* 2002), and

OnFT of orchid (Hou and Yang 2009) has been shown to cause an early flowering phenotype in Arabidopsis.

Heading date genes (*Hd*) in crop species is one of the major determinants of adaptation to different cultivation areas. It is closely related to the transition from vegetative growth to reproductive growth. Heading date 3a gene (*Hd3a*) as one of the several kind of *Hd* genes is a gene that regulate flowering time in rice and other higher plants. *Hd3a* gene was first identified as a quantitative trait loci (QTL) (Yamamoto et al., 1998, Monna et al., 2002). Protein encoded from *Hd3a* gene have proven to induce flowering of rice in a short day conditions (Kojima et al., 2002). Based on the finding of isolated *Hd3a* gene from rice by Tamaki et al., (2007), it is known that *Hd3a* is synthesized in the leaves and moves to the apical meristems to induce flowering. In addition to inducing flowering, *Hd3a* gene expressed in transgenic andigena potato cultivar produced tubers in long day's condition whereas non transgenic potato cannot produce tuber in such a condition and therefore suggest that *Hd3a* protein induce tuber formation (Navarro et al., 2011).

Genetic engineering through genetic transformation is one of the technology used to produce new varieties of plants (Hussain et al., 2013). Genetic transformation is a process to introduce genes from one organism to another. This process allows the organism of interest to contained /carried the expected characteristics/traits without changing other properties. One method to introduce genes in desire organism especially plants is through

Agrobacterium tumefaciens. This method have proven to be higher than the direct transformation method and higher gene expression stability have also been observed (Rajamuddin et al., 2016; Dai et al., 2001; Rahmawati 2006). The successful introduction of *Hd3a* genes via *A. tumefaciens* in several potato cultivars have been reported including Baraka (Bustomi 2014), Agria (Salsabila 2015), Jala ipam (Widiarti 2016) and Nooksack cultivars (Nadeak 2016). In this study we introduced *Hd3a* gene under the control of 35s *CaMV* promoter into IPB CP1 potato cultivar which is a new cultivar to induce tuber formation. Our result provide firsthand genetic/physiological information on this new potato cultivar as potential candidate among the many potatoes cultivars for tuber formation and resistance to bacteria diseases.

MATERIALS AND METHODS

Place and Time: This research was conducted at the Research Center for Genetic Resources and Biotechnology of the Bogor Agriculture University, Bogor, Indonesia, from March to December 2016.

Materials: Plant materials used in this research were the IPB CP1 potato cultivar obtained from the Research Center for Genetic Resources and Biotechnology of the Bogor Agriculture University. *A. tumefaciens* strain LBA4404 and pC1300 plasmid carrying *Hd3a* gene were used for transformation. The physical map of the T-DNA region of the pC1300- *Hd3a* plasmid is presented in Figure 1.



Figure 1: Map of *Hd3a* gene on the T-DNA in the pC1300 plasmid.

Explant preparation and plant propagation: IPB CP1 potato plants were propagated *in vitro* on MS0 medium (4.33 g/l of instant MS + 5 ml of Vitamin + 30 g/l of Sucrose + 3 g/l of gel powder and pH of 5.8) and MS2 macro medium (MS0 medium supplemented with 1x macro stock) for 4 weeks. The explants were than grown in a culture room at temperature of 24-25°C in a lighting condition of 2000-3000 lux.

Agrobacterium tumefaciens Propagation: *A. tumefaciens* strain LBA4404 carrying pC1300- *Hd3a* under the control of 35S *CaMV* promoter was cultured in liquid LB (*Luria Bertani*) medium with 50 mg/L of kanamicin, 25 mg/L and of refampicin in dark condition at temperature of 28°C for 12 hours until a OD₆₀₀ optical density of *Agrobacterium* culture of 0.4-0.5 was obtained.

Genetic transformation of IPB CP 1 potato cultivar: Prior to transformation, internode were grown in pre-

culture medium (4.33 g/l of instant MS +0.1 g/l of Myo inositol + 16 g/l of Glucose + 2 mg/L of 2, 4-D + 3 mg/l of BA + 5 mg/l of vitamin + 2.5 g/l of Gelrite pH of 5.8) in dark room for 12 hours. Cultures of *A. tumefaciens* with OD₆₀₀ = 0.5 were centrifuge at a speed of 6000 rpm for 15 min and the pellet was than dissolved in liquid of co-cultivation medium. *A. tumefaciens*- mediated genetic transformation was done through co-cultivation. Internode with of size 0.5–1 cm combine with pellet of *A. Tumefaciens* were put into co-cultivation liquid medium and so shaken for 10 min at room temperature. The Explants were than dried on sterile tissue paper for 10 min and grown on solid co-cultivation medium (4.33 g/l of instant MS + 0.1 g/l of Myo inositol + 16 g/l of glucose + 2 mg/L of 2, 4-D + 3 mg/l of BA + 5 mg/l of vitamin + 2.5 g/l Gel red + 20 mg/L Asetosiringon, pH 5.8) in dark room condition for 3 days.

Regeneration, propagation and selection of shoots: after 3 days on solid co-cultivated medium, the explants were rinsed in both sterile water and 100 mg/L Cefotaxime for 5 and 10 min respectively and then dried on sterile tissue paper for 10 minutes. Dried explants were planted in M4 shoot regeneration medium (4.33 g/l of MS instant + 30 g/l of sucrose + 0.2 g/L of Kasein hidrolisat + 0.1 g/l of Myo inositol + 3 g/L gel red + 3 mg/L BA + 2 mg/L IAA + 1 mg/L of GA3 + 5 mg/L MS vitamin + 100 mg/L Cefotaxime, pH of 5.8). Explants were grown in media without hygromycin for 2 weeks and then transferred in M4 medium containing hygromycin. With the appearance of shoots, the explants were sub-cultured on MS medium and subsequently reproduced and used for putative transgenic plant conformation. Optimization of transformed IPB CP1 explants potato cultivar (transgenic) were selected on medium containing hygromycin antibiotic. The untransformed IPB CP1 potato cultivar (non-transgenic) plant was used as control.

Identification of putative transgenic with PCR: In order to identify putative transgenic plants, we firstly isolated the genomic DNA. Isolation of genomic DNA was done using the modified method of Suharsono (2002). Isolated DNA was then amplified using Polymerase Chain Reaction (PCR). PCR was performed using promoter specific 35S-F primer (5'-CCA AGC TCT ATC TGT CAC TTC ATC-3') and specific transgenic (potato plant containing *Hd3a* gene) *Hd3a*-R primer: (5'-CTA GGG GTA GAC CCT CCT GCC-3'). The PCR result was checked by electrophoresis using 0.7-1% agarose gel in buffer solution 1 x TAE buffer (40 mM Tris-acetate, 1mM EDTA) at a volt of 100 for 30 minutes (Li et al 2011). DNA was visualized using UV light after soaking the gel in 0.5 µg/mL Ethidium bromide (EtBr) for 10 min. PCR also was done with the same DNA samples using *actin* primer *Act*-F (5'-ATGGCA GATGCCGAGGATAT-3') and *Act*-R (5'-CAGTTG TGCGACCACTTGCA-3') as internal control of the potato DNA.

RESULTS AND DISCUSSION

Transformation of IPB CP1 potato cultivar: Transformation optimization result on selection media containing 15 mg/L of hygromycin showed that transformant (transgenic) potato callus partially survive (Figure 2a) while the non-transformant did not survive (Figure 2b). The concentration of the hygromycin antibiotic used in this study was slightly higher than previous research on putative Atlantic transgenic potato cultivar (10 mg / L; Manguntungi 2014). IPB CP1 potato cultivar is a physically modified version of the Atlantic potato cultivar and so the necessity to optimize the callus induction medium for selecting the transgenic plant which is resistant to hygromycin antibiotic is essential. In this study, we used transformed plant to optimize selection and used non-transformed plant as control.

Integration of *hpt* gene into the T-DNA of pC1300 containing *Hd3a* gene into the IPB CP1 cultivar was to enable the expression of *hygromycin phosphotransferase* which is resistant to hygromycin thereby allowing the transformed plant to grow on media containing hygromycin. Brownish callus on the selection media indicate the oxidase enzymatic activity of *hpt* gene due to the wound created by the T-DNA integration (Yang *et al.*, 2016). Transformation was conducted using internode from 4-week old IPB CP1 potato as explants. The explants were first planted on pre-culture media for 1 day to initiate callus formation. Genetic transformation was carried out using co-cultivation medium containing acetosiringon. This co-cultivation medium containing acetosiringon was used to activate the *vir*-gene which increases the effectiveness of infection of *A. tumefaciens* (Rashid *et al.*, 2010). After the introduction of *Hd3a* gene, the explants were grown on solid co-cultivation media and stored in the dark room condition for 3 days. This dark room treatment provided an opportunity for *A. tumefaciens* to infect the plants. Both co-cultivation and callus induction medium contain plant growth regulator (PGR). Cefotaxime was added to the callus induction medium to turn off the *A. tumefaciens* living around the transformed plant.

Transformed explants were transferred to new media after every two-week to provide the plant with nutrients and hormones that help speed up buds formation. This treatment enables the explants to change shape after every two weeks on callus induction medium. The addition of plant growth regulators to the co-cultivation medium lead to callus initiation, regeneration, proliferation and elongation of plant shoots (Li *et al.*, 2007).

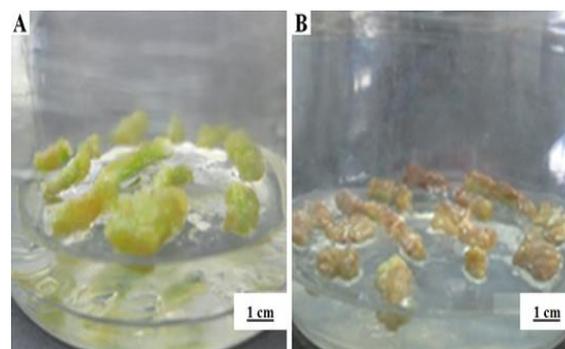


Figure-2: The condition of explants of IPB CP1 potato cultivar on regeneration and selection medium. (A) Transformed explants potato plant beginning to form callus after two weeks on regeneration and selection medium and (B) Callus plants without transformation treatment on selection hygromycin medium (browning).

The result showed formation of buds on the callus of transgenic in the 4th week on the callus induction medium (Figure 3a). In the following weeks, some of the callus further developed longer buds while some did not

survive (die) after been grown on callus induction medium containing 15 mg / L of hygromycin (Figure 3b). This indicated that the buds/shoots that survive were candidates for putative transgenic plants. Putative

transgenic plants with length 1-2cm were cut and transferred to MS medium containing 15 mg / L hygromycin for elongation and propagation (Figure 3c).

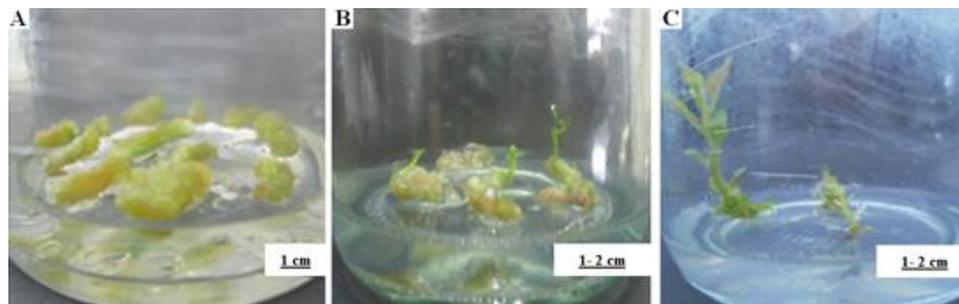


Figure 3: The development of putative transgenic IPB CP1 potato cultivar plants. (A) Callus which continues to grow in selection medium up to 1 cm, (B) Callus regenerated to form buds with measured between 1-2cm and (C) The putative transgenic plants shoots that were harvested (cut 1-2cm) and then transferred to MS medium containing hygromycin.

Genetic transformation was successfully carried out with 26 callus surviving from the total of 157 Internode explants grown on callus medium containing hygromycin. Transformation and regeneration efficiency obtained out of 26 callus hygromycin resistance were 16.4% and 23.1% respectively (Table 1). This means only 6 of the callus regenerated. The result of the transformation efficiency using internode as explants of IPB CP1 potato cv is relatively lower as compared to the result obtained from Atlantic potato cv in previous research (16.67%, Manguntungi 2014). Even though the difference in variation of transformation efficiency indicate that IPB CP1 potato cv relatively low, it suggests that genetic transformation using internode for both Atlantic and IPB CP1 potato cultivars is low. Our transformation efficiency is higher than the transformation efficiency obtained by Farhanah *et al.*, (2017) using *MmPMA* gene under the control of 35 S CaMV promoter and with different potato cultivar (3.46%; jala ipam potato cv). In another research conducted by Nadeak (2016) using the same *Hd3a* gene under the control of 35 S CaMV promoter show higher transformation efficiency of 25.21% also with different potato cultivar (Nooksack potato cv; Fatahillah *et al.*, (2016)). Variation in transformation efficiency can be attributed to different regeneration efficiency, type of potato cultivar, age of explants and the

combination different hormones (Chakravarty and Pruski, 2010; Han *et al.*, 2015).

Table 1. Transformation and regeneration efficiency of shoots of IPB CP 1 potato cv plants with gene *Hd3a*.

Observation Parameters	Total
Total number of explants	157
Number of explants that formed callus	110
Number of callus resistant to hygromycin	26
Total callus regenerated	6
Number of putative transgenic plants	6
Transformation efficiency	16.4 %
Regeneration efficiency	23.1 %

Identification of putative Transgenic Plants with

PCR: Results from the transformation showed that 6 explants could form callus and germinated successfully. The six putative transgenic shoots that survived on the selection medium were analyzed through PCR using 35S-F and *Hd3a*-R primers. This analysis was done to evaluate the integration of *Hd3a* gene into the plant genome. Results obtained from the PCR analysis showed that each of the 6 putative transgenic plants have a size of 914bp as shown in Figure 4b. *Actin* gene was used as an internal control to help identified the isolated DNA (Figure 4a). *Actin* is one type of housekeeping genes which has a stable level of expression in various tissue at all stages of plant development (Lili *et al.*, 2007).

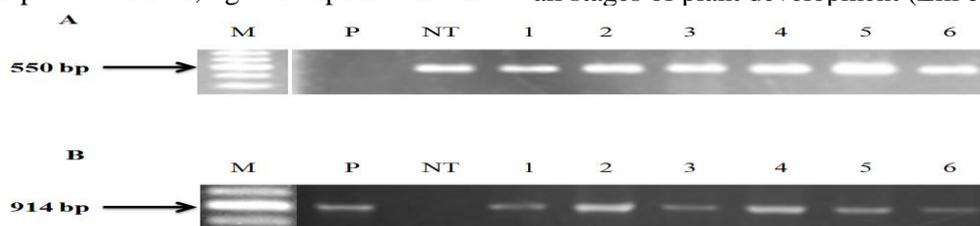


Figure 4: Analysis of *Hd3a* gene integration in IPB CP1 potato cultivar. A. PCR result of DNA using *Act*-F and *Act*-R primers and B. PCR result of transgenic DNA using 35-F and *Hd3a*-R primers. M = 1kb DNA ladder, P = pCambia1300-*Hd3a*, NT = non-transgenic IPB CP1 potato cultivar, 1-6 = DNA of transgenic IPB CP1 potato cultivar.

CONCLUSION

Hd3a gene under the control of *CaMV 35S* promoter was successfully integrated into IPB CP1 potato cultivar through *A. tumefaciens*-mediated transformation. Six putative transgenic plants survived on the selection medium containing hygromycin. Result from PCR analysis confirmed that all six putative potato contain *Hd3a* gene. This research result gives initial information on the advantage of the IPB CP1 potato cultivar as compare to other varieties been cultivated in Asia and the world at large.

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