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GENETIC TRANSFORMATION OF POTATO (*Solanum tuberosum* L.) Cv. NOOKSACK WITH *FBPase/ClRan1* GENES MEDIATED BY *Agrobacterium tumefaciens.*

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ABSTRACT

Nooksack is one of potential cultivar to be used as french fries material. *Fructosa 1.6-bisphosphatase* (*FBPase) and Citrullus lanatus Ran1(ClRan1)*genes play a very important role by increasing potato productivity. Overexpression of *FBPase* gene is able to accelerate photosynthesis whereas overexpression of *ClRan1* genesis to develop the stolon. This study aimed to introduce *FBPase/ClRan1* genes into the genome of potato cv. Nooksack used *Agrobacterium tumefaciens*. Leaf disc and internode of potato was inoculated with *A. tumefaciens* EHA105 bringing *FBPase/ClRan1* genes under control of rbsS3C promoter for *FBPase* gene and 35SCaMV promoter for *ClRan1* gene. Leaf discs have a transformation efficiency (83.33%) and regeneration efficiency (16%) better than internode with transformation efficiency (50%) and regeneration efficiency (6.66%). PCR analysis indicated that *FBPase/ClRan1* genes has been integrated into potato with size of the *FBPase/CLRan1* genes was 1047 bp. Data analysis used SPSS 16 with one way ANOVA method on the level of 95%. *FBPase/ClRan1* gene was significantly different from the photosynthesis rate, leaf number, plant height, stem diameter, the number of bulbs and tuber weight. Whereas, the wet weight and dry weight of shoots were not significantly different.

INTRODUCTION

Nooksack is one good potato cultivars to be processed into french fries. French fries have a very important role in food diversification. In addition, Nooksack has many advantages such as high-quality bulbs, white tuber flesh, high starch content, low-sugar, and resistant to bacterial wilt disease (Hoyman and Holland, 1974). However, Lauer, (1986) reported that Nooksack also has several disad-vantages such as low tuber yield and long dormancy period. Therefore, the increase in production of Nook-sack must be conducted thusit could be superior and high productivity cultivars.

One effort to increase the production of potato plants in a shorter period is through genetic transformation. Genetic transformation is a process of introduction of genes from one organism to anotherorganism by using genetic engineering techniques. In this study, genetic transformation used is *A. tumefaciens*. The use of these techniques has advantages and reasons namely; it can be conducted with simple labo-ratory equipment and the opportunity to get a single gene insertion was higher than the direct transformation techniques (Gelvin, 2003). Gene-tic transformation of potato mediated by *A. tumefaciens* have been widely applied, which was commissioned by Banerjee *et al*., (2006) on the Andigena cultivar, Khatun *et al*., (2012) on Cardinal and Heera potato cultivars and Veale *et al*., (2012) on Mnandi cultivars.

The gene that has been studied to increase the production of potato is*Citrullus lanatus Ran1 (ClRan1)* geneand fructose 1,6-bisphos-phatase (*FBPase*) gene.*ClRAN1* gene have been isolated from wild watermelon (*Citrullus lanatus)*and have been successfully performed over expression in *Arabidopsis* plants (Akashi *et al*., 2016). *ClRan1* Overexpression causes an increase in the growth of the primary root of Arabidopsis plants in drought conditions (Akashi *et al*., 2016). Over expression of *Ran1* genes derived from *Triticum aestivum* (*Ta Ran1)* can increase the cell proportion in G2 phase of the cell cycle resulting in an increase of rice and *Arabidopsis* growth (Wang *et al*., 2006). Where-as, fructose-1,6-bisphosphatase is a photosyn-thesis enzyme that is encoded by *FBPase* gene during photosynthesis (Choi *et al*., 2001). *FBPase* genes have been isolated by Tamoi *et al*., (2005). over expression of *FBPase* gene can improve photosynthesis activity, causing an increase in biomass production and paramylon accumulation on transgenicof *Euglena gracilis* compared with the wild-type (Ogawa *et al*., 2015). *FBPase* gene is also reported to have been successfully introduced into tobacco plants (Miyagawa *et al*., 2001).Increased expression of *FBPase* gene can increase the growth and photosynthetic rate of tobacco plants (Miyagawa *et al*., 2001). Consequently, it is expected that both genes can increase the production of tubers of potato plants.Therefore, this study conducted the introduction of *FBPase/ClRan1* into potato plants. *FBPase/ClRan1* gene has been cons-tructed in tandem and controlled by using *ribulose bisphosphate carboxylase small subunit* gene (rbsS3C) promoter for *FBPase* gene and promoter of 35S for *ClRan1* gene. Both pro-moters are

expected can increase *FBPase/ ClRan1* gene expression on potato thus both genes can cooperate in increasing the rate of photosynthesis and production of potato tuber of Nooksack cultivars.

RESEARCH METHODS

Materials and Methods

Materials used were potato of Nooksa-ckcultivars which obtained from Pusat Penelitian Sumberdaya Genetik dan Bioteknologi (PPSHB) IPB. Bacterial colonies used for transformation were *Agrobacterium tumefa ciens* strain EHA105 carrying pBI121 *FBPase/ClRan1* plasmid awar-ding fromProf. Akiho Yokota (*Nara Institute of Science and Technology,* Jepang). Physical map of the T-DNA region of pBI121-*FBPase/ClRan1* plasmid presented in Figure 1.

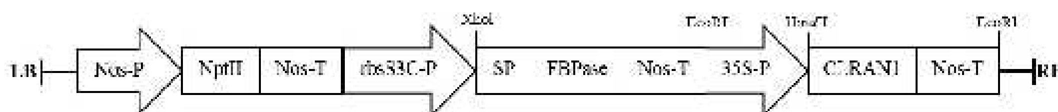

Figure 1: Mapof *FBPase/ClRan1*gene on the T-DNA in the pBI121plasmid.

In Vitro Propagation of Plant: Potato plants as in vitro propagated using single node that has one bud on macro MS_2 medium (Murashige and Skoog, 1962). The explants were planted in the MS2media for 4 weeks. The explants were grown in a culture room with a temperature of 24-25°C, withthe illumination of 1500 to 2000 lux.

Propagation of *Agrobacterium tumefaciens:* *Agrobacterium tumefaciens*was cultured for 18 hours in liquid media of Luria Bertani (LB) plus antibiotic of 100ppm kanamycin and 50ppm rifampicin, media were centrifuged at a speed of 150rpm. Bacteria were cultured at room tempe-rature in dark condition and expected to reach a value of OD_{600}amounted to 0.1-0.2.

Introductionof *FBPase/ClRan1*Gene on Potato crop: Eksplan potatoes in the form of the segment without buds and leaves were planted in pre-culture (PC) media. Explants of leaves and segments contained in the PC media stored in the dark for 1 day. The transformation was done by using co-culti-vation method using *A. tumefaciens* that carried pBI101 *FBPase/ ClRan1* plasmid.

Before soaking the explants in co-cultivation media, first *A. tumefaciens*culture centrifuged at a speed of 150rpm for 15 minutes. The precipitate obtained was resuspended in 10ml of liquid co-cultivation media containing 20ppm aceto-syringone. Ex-plants of leaves and segments were immersed in a culture of *A. tumefaciens* which had OD_{600} of 0.1-0.2 for 10 minutes while shaken. Furthermore, explants were drained on sterile tissue and left for 10 minutes and then planted in the solid co-cultivation media, which contained 20ppm acetosyringone for 48 hours at 28°C in the dark room. After two days, the explants were rinsed five times with immersion using sterile water and once with a solution of 250ppm cefotaxime. Then, the explants were drained on sterile tissue and left for 10 minutes. Furthermore, explants were grown on callus induction medium without kanamycin for 14 days. Subcultures were made every 14 days. Callus formed were transferred to selective media containing kanamycin of 100 ppm. Callus generating putative transgenic shoots were calculated to determine the efficiency of regeneration. Shoots grown from callus were cut and transferred to 1/2 MS media containing kanamycin of 100ppm. Non-transgenic explants were not transformed with *A. tumefaciens* and grown in selective media of kanamycin which was the same with ex-plants transformed with *A. tumefaciens* as a control efficiency of selective media.

DNA Isolation and Integration Analysis of *FBPase/ClRan1* Gene: Potato DNA was isola-ted using the method performed by Suharsono, (2002) were modified using CTAB buffer. Analysis of the gene integration in transgenic plants for identification of transgenic plants was performed by PCR. The composition employed in PCR reactions consisted of 500 ng of genomic DNA of *S. tuberosum*, 3pmol *primer FBPase forward,* 3 pmol *primer ClRan1 reverse*, 5µl Dream Taq™ *Green* PCR *Master Mix*, and dd H_2O to a volume of 10mL.3pmol *primer FBPase forward*, 3pmol *primer ClRan1 reverse*, 5µl Dream Taq™ *Green* PCR *Master Mix*, and ddH_2O to volume of 10µl. PCR conditions were pre-PCR with temperatures at 94°C for 2 minutes; each with 35 cycles of denaturation at 95°C for 10 seconds, annealing at 64°C for 30 seconds, extension at 72°C for 45 seconds; and one cycle for post-PCR of 25°C. *FBPase/ Cl Ran1* gene was detected using the primer of *FBPase* forward (CAAATGGCGGCG GAG CGGTAG) and *ClRan1* reverse (CAGTCTT CTGGTCCGGCAAAGCC).

Acclimatization: Potato plants that had stems and roots over 5cm were cleared and the roots formed were cut, then soaked in water containing formation hormone of roots and moved into a tray measuring 3cm x 3cm x 5cm containing cocopeat mixed with manure. After 3-week-old plants, the

plants were trans-ferred into the polybag containing the same medium as before.

Observation of Morphology: Observation ofplant morphology was performed by measu-ring and observing some of the pro-perties of plants. The properties of plants observed were stem diameter, plant height, and the number of leaves. These observations were made at random to three plants in three different polybags for each strain at the age of 4 weeks after planting (WAP). Harves-ting was carried out at the age of 11 WAP. Observation of the crop at harvest was the weight of wet canopy and shoot dry weight, tuber number and weight of tubers per plant.

Analysis of Photosynthesis rate: Analysis of photosynthesis rate was carried out at 11:00 to 14:00 on the young leaves that have grown up from the tip. The analysisusing a *portable Photosynthesis System* LICOR, type LI-6400XT (Evans and Santiago, 2014). The analysis was done randomly with each of three replications. These observations were made on 3 plants in 3 different polybags for each strain at the age of 4 WAP.

Data analysis: The data obtained were analyzed using SPSS 16 through the test of variance (ANOVA) to determine the effect of genetically modified (GMO) and non-GMO crops. If the ANOVA results give difference significantly, a further test of Significant Difference (LSD) at 95% confidence level will be performed.

RESULTS AND DISCUSSION

Introduction of *FBPase/ ClRan1*gene on Potato plant: Transformation of potato includes several stages, such us explants preparation, *Agrobacterium* infection to plants, co-cultivation, callus induction, induction of buds, shoot elongation, shoot rooting, and acclimatization. Genetic transformation is done using internodal seg-ments without buds along 0.5cm and the leaf pieces along 1cm. The addition of aceto-syringone is able to induce *vir* genes and improve the effectiveness of *A. tumefaciens* infection (Rashid *et al*., 2010).

These results indicated that the formation of calluswas immerged at the second week and regenerating wasimmersed at the fifth week after planting. Gustafson *et al*., (2006) repor-ted that the combination of auxin and trans-zeatin help the formation of callus. After the age of 14 days, the explants wererepeatedly subcultured in selective media. Non-transgenic callus growth in non-selective media was faster than the putative transgenic callus grown in the selective media. This occured because the putative transgenic callus has cultivated together with *A. tumefaciens* and had been treated with the antibiotic of cefotaxime. In addition, overgrowth in *A. tumefaciens* can also inhibit the growth and development of the callus. This problem can be anticipated by reducing the bacteria into 0.1-0.2 OD (Paserang *et al*., 2016). The low concen-tration aims to prevent bacterial overgrowth in the explant. In this study, non-transgenic callus was not treated with cefotaxime and not infected with *A. tumefaciens.*

Potato genetic transformation using *A. tumefaciens* has been successfully done using pieces of leaves and internodal segments as explant sources. The percentage of potato plant transformation efficiency by using internodal segments amounted to 50% while the leaf explants of 83.33% with an average of 66.66%.

Based on previous research, the per-centage of transformation efficiency obtained was at: 22.53% (Mardiyyah, 2015) and 25.21% (Nadeak, 2016). The high value of transformation effi-ciency can be caused by several things, including the laundering of explants were clean enough and the lack of contamination by fungi and bacteria.

In this study, the transformation effi-ciency of leaf explants had better result compared to internodal segments. This is because internodal segments often experienced browning compared with leaf explants. Tanning was caused by enzymatic reactions for their polyphenol oxidase which caused color changes to be brown.In this study, browning occurred more often on internodal segments compared to leaf explants. One effort to prevent browning in explants was to do subculture once a week. The efficiency of regeneration resulting from this research was 16.00% and 6.66% for leaf explants and internodal segments, respectively. The rege-neration efficiency of leaf explants was higher than internodal segments.

This is because internodal segments experienced browning more often than leaf explants. In this study, leaf explants had the ability to regenerate faster than internodal segments. A total of 8 callus successfully regenerated from leaf explants and 2 callus from internodal segments, but only 3 explants of them that could be shoots, i.e. 1 from internodal segments and 2 from leaf explants. These results were obtained after selective stages by using the media containing 100ppm of kanamycin. Shoots which grew from the explants in the selective media called putative transgenic shoots. Shoots which could grow in a selective medium were

transferred to 1/2 MS media which still contained 100ppm kana-mycin.

Identification of Transgenic Plants

The analysis of putative transgenic plants by PCR showed positive results as transgenic plants. Of the three putative transgenic plants, there were 2 transgenic plants and 1 non-transgenic plants. It is caused due to the escape in a selective process in which a callus formed was not covered by the media selection overall. The size of the combined base pairs of *FBPase*dan*ClRan1* gene was 1047 bp (Figure 2A). This indicated that the gene had been successfully integrated into the plant genome.

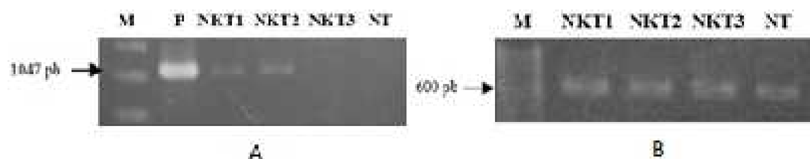

Figure 2:The results of the DNA analysis using PCR with two primer sets. (A*: FBPase*'F-*ClRan1*'Rprimer, B: using *Act*'F-*Act*'Rprimer, M = 1 kb DNA Marker; P is a positive control (plasmid); NKT1 and NKT2 are transgenic plants; NKT3: non-GMO; NT is acontrol negative (non-transgenic).

In the isolation process of plant genomic DNA was required information about the quality and quantity of plant genomic DNA. Therefore, an analysis of the integration of *actin* gene by PCR was performed. PCR results for *actin* gene produced a band with size of 600bp for all crops including control plants (Figure 2B). Actin PCR results showed that isolation of plant genomic DNA was in good quality.

Morphology Observation and Photo-synthesis Analysis: In the fourth week of vegetative observations was performed by observing plant leaves number, stem diameter, plant height and measuring the photosynthesis rate. Measure-ments and observations were conducted to determine the difference between transgenic plants and non-transgenic plants.

Table 2 Effect of *FBPase/ClRan1* gene on the photosynthesisrate, leaf number, stem diameter, and height of potato plants (S.*tuberosum* L.) Nooksack cultivars at age 4 WAP

No.	Strain	Numberof leaves (leaf)	Rod diameter (cm)	Plant Height (cm)	Photosynthesis rate (µmolCO_2 – m^{-2} – s^{-1})	Plant age (weeks)
1	NT	8.00[a]	0.18[a]	8.00[a]	14.36[a]	4
2	NKT1	14.00[b]	0.44[b]	12.80[b]	23.63[b]	4
3	NKT2	11.00[a]	0.44[b]	10.77[b]	25.72[b]	4

Note: Figures followed by the same letters and the same column are not significantly different at = 0:05

In this study, *FBPase/CLRan1*gene had significant effect on the increase in the photosynthesis rate (p=0.023), number of leaves (p=0.014), stem diameter (p=0.000) and plant height (p=0.004) on potato (*S. tuberosum* L.) cultivars Nooksack (Table 2). The increase of photosynthesis rate was allegedlycaused by overexpression of *FBPase/ClRan1* gene on potato plant. Overexpression of *FBPase* gene was capable to increase the concentration of RuBP in the chloroplasts. An increase of concentrations of RuBP in the chloroplasts can reach 30-50% (Miyagawa *et al*., 2001). The increase of photosynthesis rate have influences on the development and growth of plants. Mungara *et al.,*(2013) reported that the number of leaves in line with the number of light is absorbed for photosynthesis. Photosynthesis products that produced will be used for growth plants.

Table 3 Effect of gene *FBPase/ClRan1* against tuber number, tuber weight, wet weight of the canopy, and shoot dry weight of potato plants (*S. tuberosum* L.) Nooksack cultivars at age of 11 WAP

No.	Strain	Number of bulbs (pieces)	Weight bulbs (g)	Wet weight heading (g)	Dry weight heading(g)	Plant weight (Weeks)
1	NT	4.67[a]	13.03[a]	19.19[a]	1.11[a]	11
2	NKT1	7.33[b]	26.23[b]	21.84[a]	1.72[a]	11
3	NKT2	7.67[b]	27.19[b]	19.86[a]	1.40[a]	11

Note: Figures followed by the same letters and the same column are not significantly different at = 0:05

*FBPase/ClRan1*gene also had significant effect on the number of tubers (p=0.007) and weights (p=0.000) while the weight of the wet canopy (p=0.715). Meanwhile, shoot dry weight (p=0.280) had no significant effect on potato (*S. tuberosum* L.) Nooksack cultivars (Table 3). The Increase of tubers weight and number of transgenic potato indicated that *FBPase* gene played a role in an increase of starch synthesis (Figure 3). In addition, over expression of *FBPase* gene was capable in increasing the exchange rate of carbon in the Calvin cycle that can be used to regenerate RuBP and starch synthesis. *FBPase/ Cl Ran1*genhad no significant effect on the wet weight and shoot dry weight. Generally, photosynthesis products generated were used for root growth and to increase the tuber production.

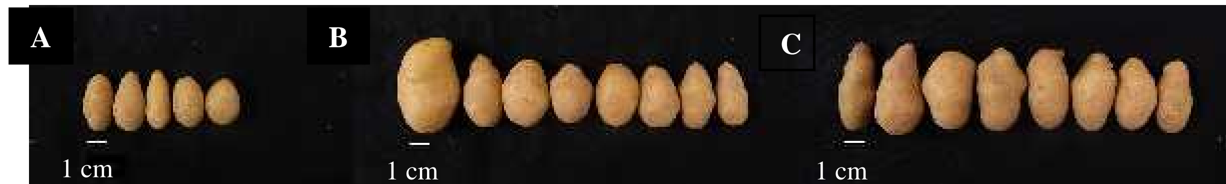

Figure 3. Comparison of the number of potato tubers containing the *FBPase/ClRan1* gene at age 11 WAP. (A) non-transgenic (NT), (B) transgenic potato (NKT1) and (C) transgenic potato (NKT2).

Comparison the root length between transgenic plants (NKT 1 dan NKT 2) and non-transgenic (NT) showed significant diffe-rences (Figure 4). This difference was caused by transgenic potato plants expressing *FBPase / ClRan1* gene. Both of these genes were able to express growth properties and plant develo-pment that is faster than non-transgenic potato. This is in accordance to what has been reported by Akashi *et al*., (2016) which ex-plained that the overexpression of *ClRan1*gene was able to increase the growth and develo-pment of the roots of the *Arabidopsis* plant.

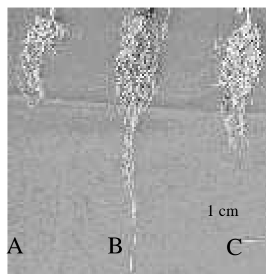

Figure 4 Comparison of the potato root morphology containing the gene *FBPase/ ClRan1* gene at age of 11 WAP. (A) non-transgenic (NT), (B) trans-genic potato (N KT1) and (C) transgenic potato (NKT2).

Genetic transformation of potato to use *FBPase* gene controlled by rbsS3Cpromoter and *ClRan1* gene controlled by 35S promoter produced transgenic potato plants. Transgenic potato plants containing *FBPase/ClRan1*genes had the photo synthesisrate and tuber production were higher than non-transgenic potato plants.

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