2-deoxyglucose selection system for *Agrobacterium*-mediated transformation of *Solanum lycopersicon* and *Nicotiana tabacum* plants

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Abstract

An alternative marker to antibiotic and herbicide genes, 2-deoxyglucose-6-phosphate phosphatase (DOG^R1) gene was used to produce transgenic tomato (*Solanum lycopersicon*) and tobacco plants (*Nicotiana tabacum*) via *Agrobacterium*-mediated transformation approach. A binary vector, pBIDOG with T-DNA cassette consists of *CaMV35S-DOG^R1-nos* was mobilized into *Agrobacterium tumefaciens* LBA4404 via electroporation and subsequently transformed into tomato and tobacco tissues. Selection of transformants of both plants was carried out by using 600 mg/l 2-deoxyglucose (2-DOG) as selection agent. 2-DOG resistant calli were regenerated into shoots and PCR analysis revealed that 75% and 71% of putative transgenic tomato and tobacco shoots were positive for DOG^R1 gene, respectively. Southern blot analysis was carried out to confirm the integration of transgene. The expected ~1.5 kb band was obtained to prove the integration of DOG^R1 gene into the plant genome. Transgenic tomato and tobacco plants were successfully regenerated without any phenotypic changes when compared to wild-type plants. Therefore, this alternative selection system can be applied to other plant transformation systems to produce transgenic plants free from antibiotic and herbicide genes, especially for important economic crops. More importantly, this selection system does not lead to any extent hazard to human and animal that subsequently will improve public acceptance of future genetically modified products.

Keywords: DOG^R1 gene, 2-deoxyglucose, transgenic tomato and tobacco, *Agrobacterium*-mediated transformation.
Abbreviations: DOG^R1_2-deoxyglucose-6-phosphate phosphatase; 2-DOG_2-deoxyglucose; IAA_indole-3-acetic acid; BAP_6-benzylaminopurine; PCR_polymerase chain reaction; MS_Murashige and Skoog medium; *CaMV35S* _cauliflower mosaic virus 35S promoter; CM_cultivation medium; *nptII*_neomycin phosphotransferase II gene; bp_base pair; kb_kilobase pair; NAA_α-naphthalene acetic acid.

Introduction

Tomato belongs to *Solanaceae* family (Vincentz et al., 2004) and was initially classified into the genus *lycopersicon* and the species *esculentum.* Based on molecular phylogenetic studies, tomato has been renamed *Solanum lycopersicum* (Peralta et al., 2006). Tomato is a very important food, and therefore it has been successfully bred to improve productivity and fruit quality, involving resistant to abiotic and biotic stresses. In addition, tomato has been widely used as a model plant and research material due to its special features such as fleshy fruit, sympodial shoots and compound leaves which other model plants such as rice and *Arabidopsis* do not have. These traits are agronomical important and cannot be studied using other model plant systems. Tomato has a very low chromosomal imbalance, high transformation frequency through *Agrobacterium*-mediated transformation of cotyledons (Barry et al., 2005; Thompson et al., 1999) and extensive cDNA and genomic libraries (Bonnema et al., 1996; Budiman et al., 2000). Tomato plants grow at high density and only take approximately three to four months for the fruit to reach full ripeness after germination, making it technically possible to obtain a large amount of tissue and study several generations in a short time.

Tobacco is a beautiful model plant system and often used for manipulating DNA in plants research. Some scholars have referred tobacco as the "white mouse" of the plant kingdom and sometime known as a keystone of solanaceous plant. The Solanaceae is a family of plant species that includes potato, tomato and tobacco with diverse phenotypes that have been exploited for a wide range of agronomic purposes (Rensink et al., 2007). Tobacco has both advantages and drawbacks. One advantage is the increased environmental and human safety. As from the aspect of containment, transgenic tobacco is either harvested before reaching maturity or tops are cut so that the transgenic tobacco does not flower. These practices can minimize the gene flow. Tobacco is a non-food crop, and thus chances of contaminating the food supply are minimal (Watson et al., 2004). One drawback of producing biologics in tobacco is that the tobacco biomass has to be processed immediately after harvest. Nevertheless, tobacco has been a useful model plant in transgenic studies. Numerous transgenic studies have been reported using the system. For example, the tobacco plant gene were altered by using the Lc regulatory gene (encodes homologous transcripts) which affects the formation of anthocyanin in plants. The flower

color of the transgenic tobacco plants was changed from light red to deep red (Chi-Chun et al., 2007).

It is important to find an effective and efficient selection system for transgenic plants which carry genes coding for important traits (AGBIOS, 2003). Selectable markers enable transgenic plants or cells to be distinguished, isolated and selectively regenerated into a whole plant after transformation. The expression of a selectable marker gene results in a product that allows the survival of the transformed cells in the presence of a selective agent but prevents the regeneration of the non-transformed cells (Brasileiro and Dusi, 1999). The most common selection markers are the genes that confer resistance to herbicides, e.g. Basta and bialaphos and antibiotics, e.g. kanamycin and hygromycin (Tuteja et al., 2012). The use of genes for antibiotic and herbicide resistance is undesired in the crops relating to food industry. As such, an intensive search for other selectable markers such as positive selectable markers has been initiated and carried out. For the commercialization of transgenic plants, the use of positive selectable marker is desirable. Replacement of antibiotic resistant genes or other marker genes eliminates the negative connotations associated with these markers and would simplify the regulatory process (Malnoy et al., 2007).

One of the potential positive selectable markers is DOG^R1 gene isolated from yeast *Saccharomyces cerevisiae* strain S288C (Randez-Gil et al., 1995). The DOG^R1 encoding 2-deoxyglucose-6-phosphate phosphatase gene can convert 2-DOG-6-phosphate derived from phosphorylation of 2-deoxyglucose (2-DOG) to non-toxic products. The 2-DOG-6-phosphate is a toxic product resulting cell death by blocking protein synthesis and subsequently reduced polysaccharide synthesis, which is very important for cell wall formation (Zemek et al., 1976). This mechanism has been applied in regeneration of transgenic plants such as tomato, tobacco, potato (Kunze et al., 2001) and oil palm (Izawati et al., 2015) by selecting the DOG^R1 transformed cells on medium containing 2-DOG.

In this study, the effects of 2-DOG on plant regeneration of tomato and tobacco were first investigated to identify the optimal concentration 2-DOG for selection of transformants. The efficient of 2-DOG as a selection agent was then evaluated by regenerating transgenic tomato and tobacco plants by *Agrobacterium*-mediated transformation approach.

Results and Discussion

Effect of 2-DOG concentration for tomato explant culture

To determine beforehand the 2-DOG concentration needed for selection of putative transformed tomato shoots, twenty cotyledons were cultivated on media supplemented with 2-DOG ranging from 0-600 mg/l (Table 1). Percentage of shoot production was calculated based on the number of shoots appeared from number of cultured explants (cross-cut cotyledons). In general, almost 100% of explants produced shoots at concentrations of 0-200 mg/l. The shoot production decreased from 60-25% at 300-500 mg/l. At 400 mg/l, 500 mg/l and 600 mg/l, shoots were produced at 35%, 25% and 0%, respectively. These concentrations are suitable to be used for selection due to the low ability of explants to produce shoots. Based on visual observation, a normal growth of callus and shoots from tomato explants was observed at concentrations of 0-300 mg/l (Fig. 2). Even though calli were produced at 400 and 500 mg/l, the shoots formed were retarded. The formation of callus and shoots was fully retarded at concentration of 600 mg/l (Fig. 2). Previous study

by Kunze et al. (2001) showed that the optimal concentration of 2-DOG to select transformed tomato explants (*Solanum lycopersicum* Mill. cv. Moneymaker) was 400-500 mg/l. The discrepancy between the results obtained in the present study and the study by Kunze et al. (2001) was probably due to the different cultivars or varieties used.

Effect of 2-DOG concentration for tobacco explants culture

As for the tomato above, 2-DOG at the concentrations ranging from 0-600 mg/l was tested on wild-type leaf explants of tobacco (Table 1). A total of 25 tobacco leaf discs were tested and the number of shoots appeared from the cultured explants was scored. At 0-300 mg/l, 100% of leaf disc produced shoots while at 500 mg/l and 600 mg/l, the shooting percentage decreased to 20% and 0%, respectively. At 400 mg/l, shoot formation was at 72% revealed that the ability of these tissues to form shoots was still high at this concentration. Therefore, the concentration is not suitable for selection of the transformed tissues because it is hard to differentiate the transformed from the untransformed tissues. The effects of different concentrations of 2-DOG is demonstrated in Fig. 2. The production of shoots from the calli was faster at 0-200 mg/l compared at 300-500 mg/l and fully retarded at concentration 600 mg/l. It can be seen by blackish colour of the tobacco explants at 600 mg/l.

Research by Kunze et al. (2001) showed that the lowest concentrations resulting in a nearly complete inhibition of regeneration of tobacco (*N. tabacum* L cv Samsun NN) was at 500-600 mg/l. Besides tomato and tobacco, 2-deoxyglucose was also tested against other explants. Kunze et al. (2001) and Masli et al. (2012) reported that the regeneration of wild-type potato and oil palm calli were fully retarded at 300-500 mg/l and 400 mg/l, respectively. Potato explants are more sensitive to 2-DOG compared to tobacco and tomato because their growth can be fully inhibited at as low as 300 mg/l. For tobacco and tomato, the effective inhibitory concentration is much higher. The results showed that different plants have different sensitivity to 2-DOG or compound used in the media. Similar behavior was also observed for other selection agents such as kanamycin and mannose. For example, 50 mg/l of kanamycin was suitable to select transformed sweet potato (*Ipomoea batatas* cv. Lizixiang) (Sheng-Jun et al., 2004), while 200 mg/l kanamycin was used to select transformed tobacco leaf disc (Mentewab and Steward, 2005). Apple young leaves required 2.5, 5 and 10 g/l of mannose for their selection (Degenhardt et al., 2006) while for the transformation of mature embryo-derived calli of Indica rice (*Oryza sativa* L.), 30 g/l of mannose was required (Penna et al., 2008).

Optimal 2-DOG concentration for selection of transgenic tomato and tobacco

For both explants (tomato and tobacco) used in this study, the possibilities of escapes are still high when the transformed explants were selected on media supplemented with 2-DOG at concentrations of 400 mg/l and 500 mg/l. Escapes are defined as plants that are regenerated on selection medium without expressing the selectable marker gene (Gallois and Marinho, 1995). Based on the result from the present study, 2-DOG at a concentration less than 600 mg/l was probably still low. Selection agent at an insufficient concentration could promote chimeras (Masani et al., 2014; Parveez et al., 2015; Izawati et al., 2015). Therefore, as a precaution to minimize escapes and to narrow down the amount of transformed tissues to be analyzed, 600 mg/l of 2-DOG was

Table 1. Effects of different concentrations of 2-DOG ranging from 0-600 mg/l on the regeneration of leaf explants of *N. tabacum* and *S. lycopersicum.*

2-DOG (mg/L)	*N. tabacum* (tobacco)			*S. lycopersicum* (tomato)		
	Total[a]	Shoots[b]	%	Total[a]	Shoots[b]	%
0	25	25	100	20	20	100
100	25	25	100	20	20	100
200	25	25	100	20	12	60
300	25	25	100	20	9	45
400	25	18	72	20	7	35
500	25	5	20	20	5	25
600	25	0	0	20	0	0

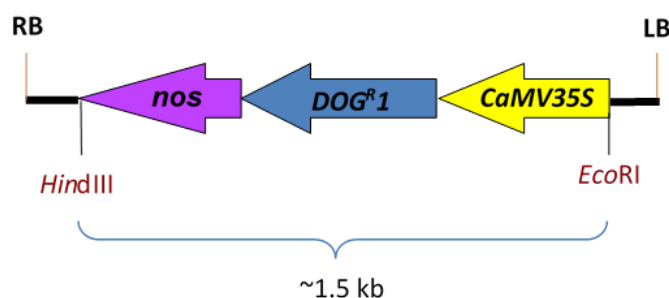[a]Total number of explants tested. [b]Number of explants developing shoots

Fig 1. Map of DNA region containing *DOG^R1* expression cassette inserted between T-DNA borders of pBIDOG (reproduced from Izawati et al., 2015). RB_right border; LB_left border; *nos*_nopaline synthase terminator; *DOG^R1*_gene codes for 2-DOG-6-P phosphatase; *35S*_CaMV35S promoter. Arrows indicate direction of transcription. The T-DNA was digested at the restriction sites (*Hin*dIII and *Eco*RI) for southern blot analysis.
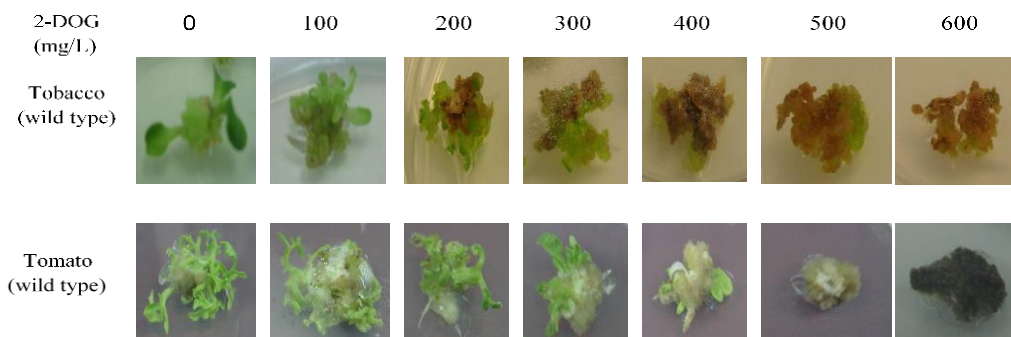

Fig 2. Effect of different concentrations of 2-DOG on tomato and tobacco tissues. Wild-type leaf explants of tobacco and tomato were cultured onto medium containing 2-DOG with concentrations ranging from 0-600 mg/l. At 0-300 mg/l, calli and shoots from tomato and tobacco explants were shown to have a normal growth. At 400 and 500 mg/l, calli were produced but shoots formed were retarded. At concentration of 600 mg/l, formation of calli and shoots was fully retarded.

chosen for selection of transformants from both tissues. Even though plants could become necrosis at this concentration, the chosen concentration is still acceptable as it will simplify the work to differentiate the transformed and untransformed tissues.

Tomato transformation, selection and regeneration

The protocol for *Agrobacterium*-mediated transformation and regeneration of transgenic tomato selected on 2-deoxyglucose medium is described below. Sterilized tomato seeds were germinated to obtain cotyledons (Figs. 3A and 3B). Cotyledons of 10-14 days old tomato seedlings were cross-cut into two pieces and used as the target tissues for the

transformation (Fig. 3C). In tissue culture, young leaf is more suitable for callus induction because it has actively young dividing cells. When the young leaves are placed in suitable callus induction medium, the cells will continue to divide and form callus. For example, Rao et al. (2004) stated that the percentage of callus induction and regeneration were higher in the young leaves compared to mature leaves of *Physalis pubescens* L. In this work, callus appeared at the cut ends of tomato explants after 4-8 weeks on MSO medium supplemented with indole-3-acetic acid (IAA) and zeatin (Fig. 3D). Transformed calli were selected from selection medium which contained 600 mg/l of 2-DOG (Fig. 3E). Resistant calli started to propagate into bigger callus (Fig. 3F). Primary shoot or shoot bud started to appear from
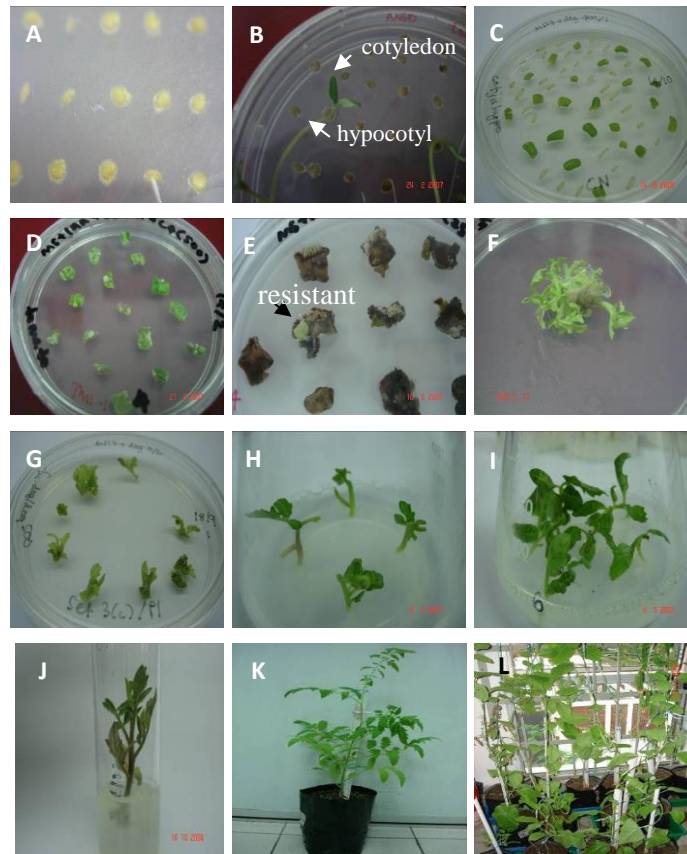
Fig 3. Development and regeneration of transgenic tomato plants. A: Germination of tomato seeds; B: cotyledons and hypocotyls; C: cut cotyledons; D: calli emerged from cut cotyledons; E: resistant calli on selection media; F: shoots appeared from calli; G and H: shoot development; I and J: root development; K and L: tomato plants in soil.
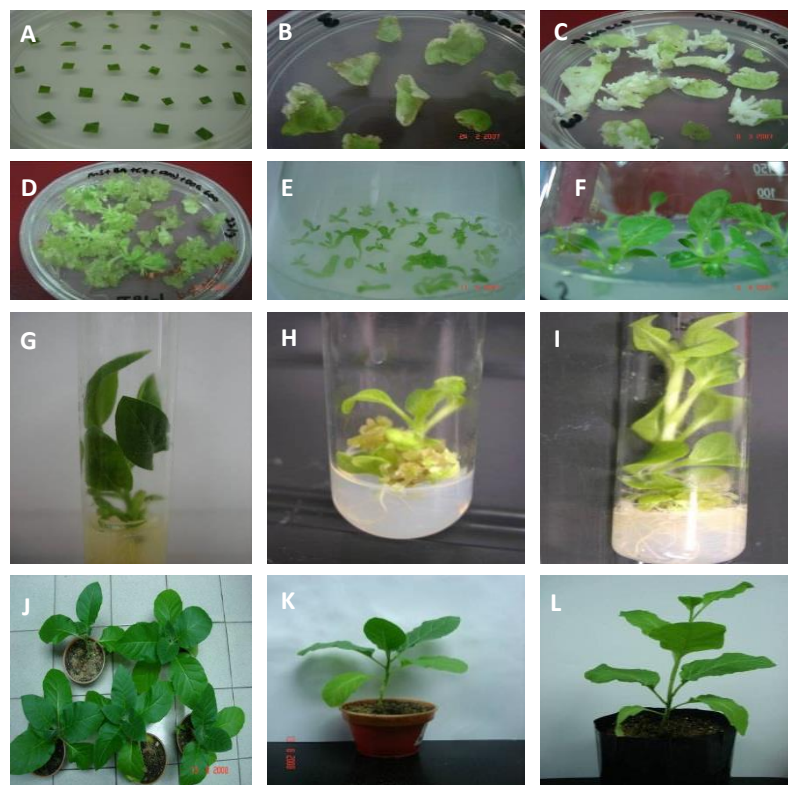

Fig 4. Development and regeneration of transgenic tobacco plants. A: tobacco leaf discs; B: expanded leaf disc and calli appeared at the edge of leaf; C and D: shoots at early formation stage; E and F: shoot development; G-I: tobacco plantlets at rooting stage; J-L: tobacco plants in soil.
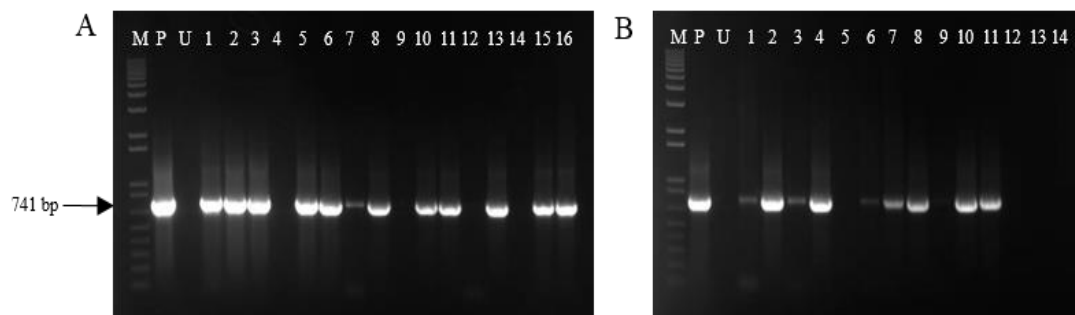
Fig 5. PCR analysis for the amplification of the 741 bp DOG^R1 transgene from putative transgenic tomato (A) and tobacco (B) plants. Lane M: 1kb plus marker; lane P: plasmid pBIDOG; lane U: untransformed control plant (wild-type plants); lanes 1-16 in (A): tomato shoots; lanes 1-14 in (B): tobacco shoots.
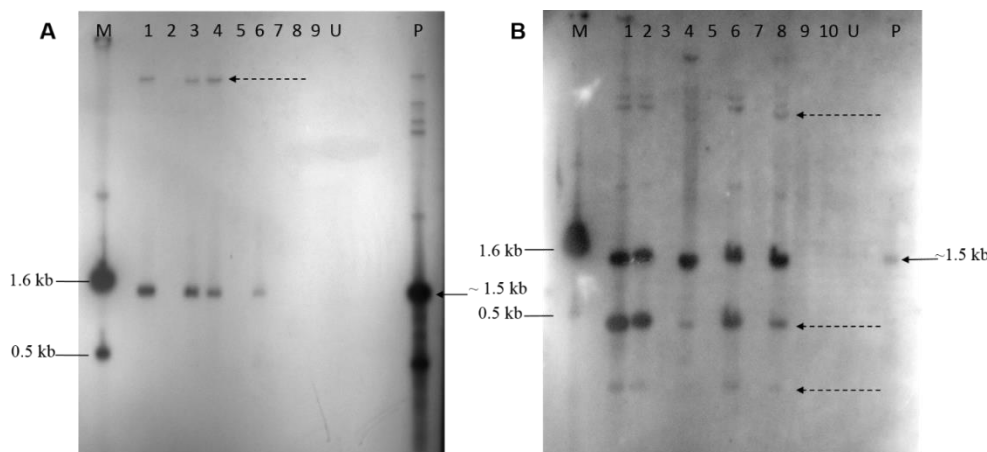

Fig 6. Southern analysis of tomato (A) and tobacco (B) genomic DNA samples which were digested with *Hin*dIII and *Eco*RI. Lane M: 1kb plus marker; lane U: untransformed control plant (wild-type plant); lane P: plasmid pBIDOG; lanes 1-9: genomic DNA from putative transgenic tomato shoots in (A); lanes 1-10: genomic DNA from putative transgenic tobacco shoots in (B).

Fig 7. Comparison between transgenic and wild-type plants. A1: Transgenic tobacco; A2: wild-type tobacco; B1: transgenic tomato; B2: wild-type tomato.

transformed callus after 12-16 weeks (Fig. 3G). The shoots were transferred onto medium without hormone to promote better growth of shoots (Figs. 3H and 3I) and roots (Fig. 3J). Plantlets with strong shoots (~15 cm height) and roots were transferred onto small pots (12 cm) covered with plastic, containing compose and vermiculite (1:1). The plantlets were maintained in growth chamber under a light/dark regime of 16 hr/8 hr at 22-25°C. After 2-3 weeks, the plantlets were repotted into polybag (20 cm) containing soil, compose and vermiculite (3:3:1) and maintained in screen house (Fig. 3K). Normal transgenic tomato plants were fully regenerated from selected 2-DOG-resistant calli in several months (Fig. 3L).

Tobacco transformation, selection and regeneration

Leaf disc transformation of tobacco is a very simple and widely used with success in many laboratories. Basically, it involves immersing the leaf discs into a liquid culture of *Agrobacterium* carrying the chosen transformation vector. The infected leaf discs (Fig. 4A) were co-cultivated onto MS medium consisted of 6-benzylaminopurine (BAP) as a growth regulator to induce calli. The medium was also supplemented with antibiotic (cefotaxime) to kill the *Agrobacterium* and 600 mg/l of 2-DOG to select

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tranformants from the untransformed cells. In plant tissue culture, calli are defined as undifferentiated mass cells that cover a plant wound (Stobbe et al., 2002). In this study, calli appeared at the cut ends of expanded tobacco leaf discs and other wounded parts of explants that occurred during the preparation of the explants for *Agrobacterium* infection (Figs. 4B and 4C). The cultures were placed under dark condition because darkness was found to be more conducive to callus production than either low or high light intensities (Teixeira da Silva et al., 2006). It took about a month for the calli to form primary shoots (Fig. 4D). Once the shoots appeared, the cultures were placed under light condition to promote photosynthetic process for better growth of the shoots (Figs. 4E and 4F). It took about two more months to develop rooted plantlets (Figs. 4G-4I). Plantlets with strong shoots and roots were transferred onto small pots (Figs. 4J and 4K). The plantlets were maintained in growth chamber under a light/dark regime of 16 hr/8 hr at 22-25°C. After 2-3 weeks, the plantlets were repotted into polybag containing soil, compose and vermiculite (3:3:1) (Fig. 4L). A total of 14 putative transgenic tobacco plants were regenerated and maintained in screen house.

Molecular analyses of putative transgenic plants

PCR analysis was performed to detect DOG^R1 gene in the genomic DNA of putatively transformed plants. The detection of expected 741 bp amplified product corresponding to the full-length DOG^R1 gene, confirmed the presence of the transgene. A total of 12 out of 16 randomly selected tomato shoot samples showed the positive band while in 14 tobacco samples tested, 10 samples were positive (Fig. 5). No band was observed in untransformed plants. Based on PCR results, the transformation frequency for tomato and tobacco is estimated at about 75% and 71%, respectively. The transformation frequency of tobacco in this study was slightly higher compared to study from Kunze et al. (2001), who demonstrated that 60-70% of 2-DOG resistant tobacco shoots harbored the DOG^R1 gene. Meanwhile for potato, Kunze et al. (2001) demonstrated that only 25% of transgenic plants were PCR positive for the DOG^R1 gene. The result shows that the transformation frequency based on 2-DOG selection system varies among plant species. For reliable verification, southern blot analysis is certainly needed to prove the integration of the transgene into tomato and tobacco genome. Therefore, randomly chosen genomic DNA of PCR-positive were digested with *Eco*RI and *Hind*III and hybridized with a *CaMV35S-DOG^R1-nos* fragment (Fig. 1). After hybridization, it was observed that only 5 out of 10 transformed DNA samples showed hybridization to the expected 1.5 kb band (Fig. 6). No signal was observed on untransformed sample (Lane U). Based on the DNA digestion profile, the ~1.5 kb band was expected to correspond to the *CaMV35S-DOG^R1-nos* fragment. This hybridization with expected band size demonstrates the integration of the transgene into plant genome. Positive signals were detected at lanes of 1, 3, 4 and 6 for transformed tomato samples (Fig. 6A) and lanes of 1, 2, 4, 6 and 8 for tobacco samples (Fig. 6B) as well as lane P for positive control plasmid pBIDOG. No signal was observed in untransformed sample (Lane U). Additional bands (dash arrows) were also observed probably due to the same *Eco*RI and *Hind*III site or caused by the partial digestion of *Eco*RI and *Hind*III site in the genomic DNA (Fig. 6).

Phenotype study of mature transgenic plants expressing DOG^R1 gene

Expression of DOG^R1 gene in transgenic plants is not expected to cause any alteration in plant metabolism due to the narrow substrate specificity of the enzyme. The 2-deoxyglucose (2-DOG) system was shown to work well in varieties of potatoes and was effective under field conditions. No phenotype differences could be found between the transgenic and the control plants (Kunze et al., 2001). This was true whereby the transgenic tomato and tobacco plants expressing DOG^R1 gene obtained in this work did not show any phenotypic differences when compared to wild-type or untransformed plants (Fig. 7)

Materials and Methods

Plant material

Tomato seeds of *Solanum lycopersicon* MT1 were surface sterilized by soaking in 20% (v/v) sodium hypochlorite solution for 20 min followed by washing in sterile distilled water for 5 times. The sterile seeds were then placed on MSO medium [4.4 g/l MS salts (Murashige and Skoog, 1962), R_3 vitamin (1 g/l thiamine, 0.5 g/l nicotinic acid, 0.5 g/l pyridoxine), 30 g/l sucrose, pH 5.9 and 8 g/l agar] and germinated in a growth chamber under a light/dark regime of 16/8 hr at 25°C. Meanwhile, *In vitro* culture plants of *Nicotiana tabacum* were cultured on MSO medium and placed under light/dark regime of 16/8 hr at 25°C.

Determination of 2-DOG concentration on wild-type explants cultivation

Tomato cotyledons from 10-14 days-old seedling were cross-cut into two pieces (~1 cm^2) and cultured onto MSIZ medium [MSO medium, 0.1 mg/l indole-3-acetic acid (IAA), 1 mg/l zeatin] with increasing 2-DOG concentrations from 100 to 600 mg/l. The explants were cultured onto fresh medium every 3 weeks and incubated under 16/8 hr (light/dark) at 25°C for formation of callus and shoots. Once shoots appeared from the callus, the cultures were transferred onto MSO medium. On other hand, *In vitro* sterile tobacco young leaves were cut into small pieces (1-2 cm^2) and transferred onto MSB medium [MSO medium, 1 mg/l 6-benzylaminopurine (BAP)] with increasing 2-DOG concentrations (100-600 mg/l) and incubated under the same condition as tomato explants for callus and shoot development. The tobacco shoots were subcultured on MSO medium without BAP for maturation.

Agrobacterium tumefaciens strain and vector

Agrobacterium tumefaciens, strain LBA4404 harboring pBIDOG plasmid (Fig. 1) (Izawati et al., 2015) containing *nptII* (neomycin phosphotransferase) gene for bacterial selection and DOG^R1 (2-deoxyglucose-6-phosphate phosphatase) gene as the plant selectable marker was used. The DOG^R1 gene was under the control of *CaMV35S* promoter.

Preparation of Agrobacterium suspension

About 50 µl glycerol stock of *Agrobacterium tumefaciens*, LBA4404 which carries plasmid pBIDOG was cultured in 20 mL LB liquid medium containing 50 µg/ml kanamycin and 50 µg/ml rifampicin and incubated for 2 days. Two ml of the *Agrobacterium* culture were transferred into fresh 18 ml LB

liquid medium and incubated for one and a half hours or until the optical density reached 0.5 to 0.6 nm prior to transformation.

Plant transformation and regeneration of transgenic plants

Tomato

Cotyledons from 10-14 days-old seedlings were cross-cut into two pieces and cultured on co-cultivation medium (CM) [MSO medium, 1 mg/l BAP, 0.1 mg/l NAA (α-naphthalene acetic acid), pH 5.8] for 1-2 days in dark condition prior to transformation. The explants were infected with *Agrobacterium* suspension by soaking for 2-3 min. After infection, the explants were dried on sterile filter paper, placed on CM medium, and then incubated at $28^{o}C$ in the dark for 2-3 days. The explants were then transferred to regeneration medium (MSIZ) supplemented with 400 mg/l cefotaxime and incubated at $28^{o}C$ in the dark for a week. Then, explants were transferred onto MSIZ medium supplemented with 200 mg/l cefotaxime and 600 mg/l 2-deoxyglucose [2-Deoxy-D-glucose Grade II (D8375), Sigma-Aldrich, St. Louis, USA] and incubated at $25^{o}C$ with 16/8 hr (light/dark). The explants were transferred to fresh medium every 3 weeks until shoots were formed. Regenerated shoots were further cultured on MSO medium containing 600 mg/l of 2-DOG for root induction. A duration of 5-6 months is required to regenerate rooted transgenic plantlets from the time of co-cultivation. Once roots were well established, the plantlets were transferred to soil for maturation.

Tobacco

Tobacco leaf discs were infected by soaking in the *Agrobacterium* suspension culture for 2-3 min. The inoculated leaf discs were dried on sterile filter paper and placed onto CM2 medium [MSO medium, 1 mg/l BAP] at $28^{o}C$ in the dark for 2-3 days. Next, the leaf discs were cultured onto CM2 medium supplemented with 400 mg/l of cefotaxime for a week. The explants were then transferred onto CM2 medium supplemented with 200 mg/l of cefotaxime and 600 mg/l 2-deoxyglucose (2-DOG) and cultured at $25^{o}C$ in dark condition. Cultivation of explants was performed every 3 weeks until calli and shoots were formed. To induce shoot elongation and rooting, the explants were cultured onto MSO medium containing 600 mg/l of 2-DOG. A duration of 3-4 months is required to regenerate rooted transgenic plantlets from the time of co-cultivation. Once roots were well established, the plantlets were transferred to soil compost and grown to maturity.

Genomic DNA extraction, Polymerase Chain Reaction (PCR) and southern blot analyses.

Genomic DNA was isolated from putative transgenic tomato and tobacco shoots by using the modified CTAB method (Doyle and Doyle, 1990). PCR analysis was performed to verify the presence of $DOG^{R}1$ gene in the genomes of putative shoots. DNA was amplified using the $DOG^{R}1$ gene primers (DOG^{R}1: 5'-ATGGATCCCCATGGCAGAATTTT CAGCTGATCTAT G-3' and DOG^{R} 2: 5'-ATGTCG ACTACTCAGGCCCTTGTCAAAGGGTTG-3'). Reaction conditions were as follows: the reaction mixtures were initially heated at $95^{o}C$ for 5 min. Then the amplification was performed in 10 cycles at $95^{o}C$ for 1 min, $70^{o}C$ for 1 min and $72^{o}C$ for 1 min. The annealing temperature was reduced by $1.7^{o}C$ in each cycle. The reaction was continued in 25 cycles

of $95^{o}C$ for 1 min, $53^{o}C$ for 1 min and $72^{o}C$ for 1 min. The reaction mixtures were subjected to a final extension of $72^{o}C$ for 5 min. PCR products were separated by electrophoresis in 1% (w/v) agarose gels at 100V and detected by ethidium bromide staining.

For southern blot analysis, 30 µg of genomic DNA from putative transgenic plants were digested with *Eco*RI and *Hin*dIII and separated on 1% agarose gel in 1XTBE buffer at 30V overnight. The digested DNA was transferred onto nylon membrane (Amersham Hybond-N^{+}) by capillary blotting (Sambrook et al., 1989). The nylon membrane was incubated overnight at $50^{o}C$ in hybridization buffer (0.5M Na_2HPO_4, 0.5M NaH_2PO_4, 0.5M EDTA pH 8.0, 7% SDS+ α^{32}P-dATP labelled probe). The radioactive labelled probe was prepared by using 1.5 kb DNA fragment *CaMV35S-DOG^{R}1-nos* extracted from pBIDOG plasmid by digestion with *Eco*RI and *Hin*dIII (Fig. 1). The membrane was washed with 2X SSC/0.1% SDS at $50^{o}C$ for 10 min followed by 1X SSC/ 0.1% SDS at $50^{o}C$ for 10 min. The membrane was exposed to an X-ray film (Kodak Photo Film) at $-70^{o}C$ for 7 days. The film was visualized using the standard developer and fixer solutions (Kodak).

Conclusion

We successfully produced transgenic tomato and tobacco plants *via Agrobacterium*-mediated transformation using $DOG^{R}1$ gene as a selectable marker. We achieved moderate and acceptable transformation frequencies for both transgenic plants exhibiting normal phenotype demonstrated that the efficient of 2-DOG selection system. As an alternative to herbicides and antibiotics marker, this positive selection employing $DOG^{R}1$ gene can be used to develop a better selection system for other important crops such as rice and maize transformation. Recently, we have used 2-DOG selection system for oil palm transformation (Izawati et al., 2015) and experiments to improve the transformation rate are being intensively carried out.

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