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Agrobacterium tumefaciens Mediated Transformation of 
rolC::Hd3a-GFP in Black Rice (Oryza sativa L. cv. Cempo Ireng) to Promote Early Flowering

Yekti Asih Purwestri\textsuperscript{a,b},*, Resta Dewi Komala Sari\textsuperscript{a}, Lisa Novita Anggraeni\textsuperscript{b},
Aries Bagus Sasongko\textsuperscript{b}

\textsuperscript{a}Research Center for Biotechnology, Universitas Gadjah Mada Jl. Teknika Utara, Bantul Yogyakarta 55281, Indonesia
\textsuperscript{b}Faculty of Biology, Universitas Gadjah Mada, Jl Teknika Selatan Sekip Utara, Yogyakarta, 55281, Indonesia

Abstract

The purpose of this study was to transform rolC::Hd3a-GFP by Agrobacterium tumefaciens on black rice (Oryza sativa L. cv. Cempo Ireng). The callus formation were induced from scutellum using two different media, 2N\textsubscript{6} and 2,4-dichlorophenoxyacetic acid (2,4-D). PCR analysis using specific primers for Hd3a and hpt was performed to determine the stability of rolC::Hd3a-GFP construct before co-cultivation of callus and Agrobacterium tumefaciens. This study indicated that black rice callus induction on 2N\textsubscript{6} medium responded faster than that on MS 2,4-D medium and generated friable calli for Agrobacterium-mediated transformation. The screening result showed that several calli were competent to regenerate on medium containing hygromycin and demonstrated Hd3a insertion gene.

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Keywords: Black rice (Oryza sativa L. cv. Cempo Ireng); Hd3a; hpt; 2N\textsubscript{6} medium; 2,4-dichlorophenoxyacetic acid; Agrobacterium-mediated transformation.

*Corresponding author. Tel.: +62 274 545 187; fax: +62 274 580 839; cell phone: +62 852 8241 5959
E-mail address: yekti@ugm.ac.id
1. Introduction

Black rice (Oryza sativa L. cv. Cempo Ireng) is rice that has a high nutrient content such as high fiber, rich in iron, amino acids and anthocyanin. Extract black rice is rich in anthocyanins that identified containing cyaniding-3-glucoside and peonidin-3-glucoside. Anthocyanin extract is reported to improve lipid profiles in apolipoproteinE-deficient mice by lowering levels of triglycerides, total cholesterol and non-HDL cholesterol. The high benefits of black rice is not comparable with relatively low availability in the market. This is because black rice cultivation is considered quite long compared to white rice planting. The productivity of black rice about 4.5 t · ha⁻¹ and rice crop is harvested at 5 mo. To increase the productivity, cropping period has to be shorten through genetic engineering methods by author. Gene transformation is one way of genetic engineering methods that play a role in reducing or adding genes in a genome sequence of an individual. Flowering is one of the life cycle of the plant to produce flowers, fruits, and seeds.

Hd3a (Heading date 3a) were identified by Quantitative Trait Locus (QTL), which showed flowering in rice under short-day conditions. Currently Hd3a and RFT1 (rice Flowering Locus T1) have been known as a flowering signals under short-day conditions and long-days, respectively. Previous research in rice cv. Japonica showed that Hd3a expressed under the control of rolC promoter exhibited early flowering phenotype. Hd3a expression under the control of rolC promoter is expected to accelerate flowering in black rice cv. Cempo Ireng to improve the rice productivity to support national food security. The purpose of this research is to study the process of transformation rolC::Hd3a-GFP mediated by Agrobacterium tumefaciens into black rice callus to promote flowering.

2. Materials and methods

2.1. Seed material and culture media

Black rice seeds cultivars Cempo Ireng were obtained from the Research Agency for Agriculture Technology, Yogyakarta, Indonesia. The media used for bacterial and plant tissue culture are listed in Table 1.

<table>
<thead>
<tr>
<th>Nomenclature</th>
<th>Definition</th>
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<td>s</td>
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Table 1. Media used for bacterial culture, tissue culture and transformation of black rice cv. Cempo Ireng

<table>
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<tr>
<th>Culture Medium</th>
<th>Composition</th>
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<tr>
<td>Agrobacterium culture</td>
<td>AB media: glucose 1.25 g, agar 3 g/225 mL, 12.5 mL AB buffer (60 g · L⁻¹ K₂HPO₄, 26 g · L⁻¹ NaH₂PO₄·2H₂O), 12.5 mL AB salt (20 g · L⁻¹ NH₄Cl, 6 g · L⁻¹ MgSO₄·7H₂O, 3 g · L⁻¹ KCl, 0.264 g · L⁻¹ CaCl₂·2H₂O, 0.05 g · L⁻¹ FeSO₄·7H₂O)</td>
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<tr>
<td>Callus induction</td>
<td>2N₆ [4 g N₆ powder/L, 1 mL · L⁻¹ N₆ vitamin (2 g · L⁻¹ glycine, 1 g · L⁻¹ thiamine-HCl, 0.5 g · L⁻¹ nicotinic acid, 0.5/L pyridoxine-HCl), 30 g · L⁻¹ sucrose, 100 μL · L⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D) (20 mg · mL⁻¹), 2.88 g · L⁻¹ L-proline (adjust pH 5.7)]</td>
</tr>
<tr>
<td>Co-cultivation</td>
<td>4 g · L⁻¹ N₆ powder, 2 mL · L⁻¹ FeEDTA, 1 mL · L⁻¹ MS vitamin, 30 g · L⁻¹ sucrose, 10 g · L⁻¹ glucose, 200 μL · L⁻¹ 2,4-D (20 mg · mL⁻¹), 1 mL · L⁻¹ acetosyringone (200 mg · mL⁻¹) 6 g · L⁻¹ agarose (adjust pH 5.7)</td>
</tr>
<tr>
<td>Plant Regeneration</td>
<td>50 mL · L⁻¹ R2 macro solution [80 g · L⁻¹ KNO₃, 6.7 g · L⁻¹ (NH₄)₂SO₄, 5 g · L⁻¹ MgSO₄·7H₂O, 3 g · L⁻¹ CaCl₂·2H₂O, 5.46 g · L⁻¹ NaH₂PO₄·2H₂O], 1 mL · L⁻¹ R2 micro solution [160 g · L⁻¹ MnSO₄·4H₂O, 220 mg · L⁻¹ ZnSO₄·7H₂O, 12.5 mg · L⁻¹ CuSO₄·5H₂O, 600 mg · L⁻¹ H₂BO₃, 12.5 mg · L⁻¹ Na₂MoO₄·2H₂O], 2 mL · L⁻¹ FeEDTA, 1 mL · L⁻¹ MS vitamin, 30 g · L⁻¹ sucrose, 30 g · L⁻¹ sorbitol, 10 g · L⁻¹ agarose (adjust pH 5.6 to pH 5.8)</td>
</tr>
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</table>
2.2. Methods

2.2.1. Black rice callus induction

Mature seeds of black rice cv. Cempo Ireng were dehusked and sterilized by immersion in 70% ethanol for 30 s followed by soaking 20 min in 10% (v/v) of sodium hypochloride (10% active chlorine). Then they were washed three times with sterile water. The sterilized seeds were immersed in sterilized water for 1 h and cultured with the scutellum pointing upward on callus induction 2N6 and MS 2.4-D medium at 28 °C in light condition. Callus was sub-cultured to 2N6 medium for 3 d before co-cultivation with Agrobacterium tumefaciens.

2.2.2. Determining stability of rolC::Hd3a-GFP construct in Agrobacterium tumefaciens

Several single colony of Agrobacterium tumefaciens was grown on AB medium and using as template for amplification Hd3a and hpt gene. PCR colony was performed using thermal cycler (Master Cycler Personal Eppendorf) with 20 µL of PCR reaction mixture containing Ex Taq polymerase (TAKARA). Primers used in this study were: hpt (Forward: GAGCCTGACCTATTGCATCTCC, Reverse: GCCCTCAGAAGAAGATGTTGG, Hd3a (Forward Primer: AGCCCAAGTGACCCTAACCT, Reverse: GTACACTGTCTGACGCCCCA). PCR cycling condition were denaturation at 96 °C for 2 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min and final extension at 72 °C for 10 min. Amplified products (Hd3a and hpt genes) and 100 bp marker were separated by 1% agarose gel electrophoresis (Sigma).

2.2.3. Transformation of rolC::Hd3a-GFP construct into callus cells mediated by Agrobacterium tumefaciens

A. tumefaciens strain GV3010 containing p2K1 binary vector rolC::Hd3a-GFP was streaked out on AB medium supplemented with 50 mg/L kanamycin and hygromycin, and grown at 28 °C for colonies to appear. The Agrobacterium culture suspension for co-cultivation was prepared in 20 mL MSL medium and grown until reach of 0.008 OD600 to 0.01 OD600.

After four weeks of culture, the proliferating calli derived from scutellum were sub-cultured on fresh callus induction medium for 4 d. Further, the calli that showed a compact and nodular appearance were immersed in an Agrobacterium suspension before transfer to solid co-cultivation medium. After co-cultivation, the infected calli were washed with sterile water containing 250 mg · L⁻¹ carbenicillin to kill Agrobacterium and the transformed calli were transferred to selection medium contained hygromycin 50 mg · L⁻¹. The transformed calli in the selection medium were cultured at 28 °C in light for 2 wk.

Fresh, healthy-looking hygromycin-resistant calli were transferred to regeneration medium. The calli in regeneration medium were incubated at 28 °C in light for 2 wk, and then transferred to fresh regeneration medium.

2.2.4. Screening calli

DNA of calli grown in selective media containing hygromisin were isolated and were used as a template for amplification of hpt gene using specific primers in order to determine the insertion of gene of interest.

3. Result and discussion

Selection of Agrobacterium tumefaciens harboring rolC::Hd3a-GFP binary vector was performed by growing bacterial colonies in solid LB medium containing antibiotics kanamycin and hygromycin 50 mg · L⁻¹. It is very important to determine the stability of gene insert before Agrobacterium-mediated transformation into black rice calli. Colony PCR analysis demonstrated that both of Hd3a and hpt genes were expressed in Agrobacterium tumefaciens (Fig. 1).
Callus initiation is the first step in the process of Agrobacterium-mediated genetic transformation of rolC::Hd3a-GFP. Explants used in this study were scutelum that has embryonic properties and protected by the seed endosperm. Therefore, scutelum is sterile. In this study, two callus induction media, 2N6 medium and 2,4-D medium were used to obtain embryonic calli of black rice. Both of these mediums have different nutritional composition. Callus growth appeared after 11 d after culture. Callus induction on 2N6 medium responded faster than that on MS medium 2,4-D.

The present study is the first to report on Agrobacterium-mediated genetic transformation in black rice. Scutellum-derived calli from mature black rice seeds were co-cultivated with Agrobacterium tumefaciens that carried p2K binary vector rolC::Hd3a-GFP, which contained the genes for hygromycin resistance (hpt). After co-cultivation using Agrobacterium tumefaciens, calli were able to grow on regeneration selection medium containing hygromycin 50 mg/L (Fig. 2). Regeneration calli into the plantlet are in progress. The gene of interest were inserted in the regeneration calli, is indicated in author’s result (Fig. 3).

Fig. 2. Calli initiation and genetic transformation of rolC::Hd3a-GFP using Agrobacterium tumefaciens. (i) callus were generated from rice seed scutellum; (ii) calli were subculture into 2N6CO media; (iii) co-cultivation of calli and Agrobacterium tumefaciens; (iv) calli were able to proliferate when culture on medium R2R containing hygromycin
Fig. 3. RT-PCR analysis showed three independence lines of transgenic rice plants overexpressing rolC::Hd3a-GFP. hpt (Hygromycin phosphor transferase) indicated the gene insertion and ubiquitin as internal control. WT (Wild Type)

4. Conclusion

This study indicated that black rice callus induction on 2N6 medium responded faster than that on MS medium 2,4-D and generated friable calli for Agrobacterium-mediated genetic transformation. Several calli showed the ability to grow in selective media containing hygromycin indicating the insertion of rolC::Hd3a-GFP. Further research for selection of calli which competent to regenerate shoots on regeneration medium and the stable rolC::Hd3a-GFP expression in plantlets need to be optimized.

References