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AND 5th KBI CONGRESS
JULY 27-29, 2010


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Theme:
"Biotechnology: Breakthrough for the Future of Industrial
Challenges in Developing Countries"

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RESEARCH OF ENVIRONMENT

1. Vivtri Dewi Prasasty
   Isolation And Characterization of Bacterial Lipase from Urban Rivers in Jakarta

2. Dewi Fitriani
   Site-Directed Mutagenesis of Glu-269 L-Arabinose Isomerase from Local Geobacillus stearothermophilus Isolated from Tanjung Api, Poso, Indonesia

3. Saptowo J. Pardal
   The Expression of Parthenocarpic Gene, DefH9-iaaM on Transgenic Tomato Lines

4. I G.P. Wirawan
   Isolation of Genes by Using Transposon and T-DNA Tagging Methods

5. Agung Surono
   Flower Development of 4 Varieties of Phalaenopsis orchid After Hand Self- Pollination.

6. Endang Semiarti
   High frequency of Agrobacterium-mediated Genetic Transformation of Indonesian Black Orchid (Coelogynae pandurata) Induced by Acetosyringone

7. Achmad Himawan
   Flower Development of 4 Varieties of Phalaenopsis orchid After Hand Self- Pollination

8. Rindang Dwiyani
   Improvement of Genetic Transformation Technique in Vanda Tricolor Orchid Using Acetosyringone

9. Ariandana Wantho
   In Vitro Embryogenic Callus Induction and Regeneration of Anthurium plowmanii Using 2,4-D and BAP

10. Mirni Lamid
    Degradation of Dry Matter And Crude Fiber of Rice Straw By Addition Bacteria Xylanolytic

11. Dian Indratmi
    Aplication Debaryomyces sp and Scizosaccharomyces sp With Adjuvant for Biocontrol Disease on Mango

12. Diani Fatmawati
    Genetic Diversity of Sumatra Native Cattle Based on Cytochrome b Gene

13. Ahmad Wahyudi
    Administration of Lignocellulose Degrading Fungi Isolated from Herbivore’s Gastrointestinal Tract on Fiber Degradation

14. Hany Handajani
    Experimental Studies on the Growth of Azolla as Biofertilizer for Acid Water System

15. Ima Yudha Perwira
    Effect of Supplementation of Medicinal Plant Extract to Fish Sperm Extender Solution (Ca-F-Hbss)
16. Sri Dwi Hastuti
   Effect of Poly I:C and LPS Injection on Immune Responses of Redclaw (*Cherax quadricarinatus*)

17. Sri Wahyuni
   Rub Preparations Comparative Preparation for Improving the Quality Femur Preparations Mounted

18. Sunarto
   Processing of Municipal Solid Waste in Temporarily Disposal Site as the Main System to Reduce Green House Gases

19. Rr. Eko Susetyorini
   Study on Plant Habitat Beluntas (*Pluchea indica*) Compounds and Content on Leaves Beluntas

20. Noor Harini
    Study on Plant Habitat Beluntas (*Pluchea indica*) Compounds and Content on Leaves Beluntas

21. Sukardi
    The Influence of Concentration of Papain Enzyme and Long of Fermentation to Physical and Chemical Caracteristic of Coconut Oil (*Cocos nucifera* Linn)
High frequency of Agrobacterium-mediated Genetic Transformation of Indonesian Black Orchid (*Coelogyne pandurata*) Induced by Acetosyringone*

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Abstract

High frequency transformation of Indonesian Black orchid (*Coelogyne pandurata* Lindley) protocorms (developing orchid embryos) has been obtained using disarmed Ti plasmid containing the coding region of a hygromycin phosphotransferase gene (HPT) as a selectable marker. A flowering time gene of rice *Heading Date3a (HD3a)* under the control of Cauliflower Mosaic Virus (CaMV) 35S promoter was also inserted in plasmid PZP binary vector in *Agrobacterium tumefaciens* strain GV3101. To get high efficiency of T-DNA transfer from Agrobacterium into plant, acetosyringone (AS), a wound response molecule was added into preculture and cocultivation medium. After 2 weeks precultured and 30 minutes cocultivated in half strength New Phalaenopsis (NP) liquid medium with and without AS, the result showed that the frequency of transformation was highly improved into ranged from 17 to 42.4 percent when AS was added. Without AS, the frequency of transformation was 1.02 percent. The selected transformant protocorms were maintained on NP medium with addition of 3 mg.l⁻¹ of 2-isopenthenyl adenine (2-iP) and 0.15 of Naphthalene acetic acid (NAA). Positive transformants were confirmed by polymerase chain reaction (PCR) amplification of inserted genes.

Keywords: acetosyringone, Agrobacterium tumefaciens, protocorms, transformation
INTRODUCTION

Orchid is very popular ornamental plant in the world. There are some wild orchid species in Indonesia. One of Indonesian orchid that has a specific character (large size of flower, greenish sepal and petals, and black labellum) is Black orchid (*Coelogyne pandurata* Lindley). This orchid is an endemic orchid of some Indonesia Province, such as East Kalimantan and Papua.

The general problem in growing orchid is their late flowering, including Black orchid. Endogenous genetic factor and environmental signal control the time of flowering. One of the environmental signals is photoperiod. Genetic controls mechanisms of photoperiodic response of flowering have been analyzed using some model plants, i.e. Arabidopsis and Rice. Kojima et. al (2002) has been isolated and detected one of genes that control Flowering Time of Rice through photoperiod pathway, e.g Heading date 3a (Hd3a). Overexpression of this gene could induce early flowering in both Rice and Arabidopsis. Since rice is a monocot, overexpressing Hd3a gene to orchids is hopefully worked to induce early flowering in orchids.

To get the optimal condition of transformation, the use of a wound response molecule such acetosyringone (AS) was often used in some experiments, i.e Sheikholeslam and Weeks (1987) in Arabidopsis that increased the efficiency of transformation from (2-3)% become (55-63)%. Atichart *et al.* (2007) used 200 uM AS in transformation system of *Dendrobium secundum* orchids got about 38% transformation efficiency. Since, the frequency of transformation in orchids usually was relatively low, so it worth to try to use the addition of AS into Agrobacterium culture prior to transformation into black orchid system.

MATERIALS AND METHODS

Plant materials and culture condition
Mature seeds and 4-month-old protocorms of black orchids were used as plant materials. The Protocorms were given by Mr. Wirakusumah (the owner of Edward and Frans
Orchids Nursery, East Java). Seeds from fully ripening fruit (five-months-old fruit) were sown on New Phalaenopsis (NP) (Islam et al., 1998) with addition of 150 ml.1\(^{-1}\) coconut water. The in vitro cultures were incubated at 25\(^{\circ}\)C with 1000 flux continuous light. The growth of protocorms, shoots and plantlets were examined every week. Two weeks old protocorms were used as plant materials for transformation.

**Genetic Transformation**

Genetic transformation of plasmid 35S::Hd3a and pPZP vector into orchid was carried out according to the method of Semiarti et al. (2007). Except that the liquid medium that was used to rinse the protocorm was half strength of VW medium and 300 mg.1\(^{-1}\) Cefotaxim. SIM (*Shoot Induction medium;* 0.15μM NAA+ 5μM 2iP) supplemented with 20 mg.1\(^{-1}\) Hygromycin for selecting independent transformant. Frequency of transformation was decided by the rasio of the number of survive protocorms per total number of transformed protocorms. Fig. 1 shows the structure of T-DNA used in this study.

![T-DNA structure diagram](image)

Fig. 1. T-DNA structure containing 35S::Hd3a used for transformation. LB, Left border; RB, Right Border, 35S: CaMV promoter; Hd3a:: Heading date 3a gene; HPT: Hygromycin phosphotransferase; Tnos: Nos terminal.

**RESULTS AND DISCUSSION**

**Morphology of the black Orchid**

The black orchid (*C. pandurata*) is epiphytic sympodial orchid. Some pseudobulbs grow parallel with two leaves each. Five to- seventh flowers were arranged in a raceme, fragrance, each flower is 7-12 cm in diameter. Sepals and petals are green and the labellum (lip) is black. (Fig. 2)
Frequency of genetic transformation of black orchid using A. tumefaciens after Acetosyringone treatment

The transformation results of p35SHd3a show that without any AS addition in Agrobacterium culture in both pre-culture medium and cocultivation medium, the frequency of transformation is about 17% (78 of 470 protocorms are hygromycin resistant). Addition of AS in 25 mg.l⁻¹ did not affect the frequency of transformation of Hd3a gene into black orchid protocorms. But, addition of (50-75) mg.l⁻¹ AS, significantly increased the frequency of transformation into (42-49)%. The optimal concentration of AS addition is 75 mg.l⁻¹, that produced the highest frequency of transformation (49%). When we added the AS more up to 100 mg.l⁻¹ the frequency of transformation decreased up to 11%, that lower that in AS-free condition (Fig.3 and Table 1). PCR analysis confirmed the insertion of Hd3a cDNA into the transforman of black orchid, that a 100-bp of Hd3a fragment were amplified from the transformants (Fig. 4). The PCR analysis using HPT gene is still in progress. From this result, it is also shown that the frequency of transformation in the black orchid is higher than that in other natural orchid, *Phalaenopsis amabilis* and become 16.6% after addition of 100 mg.l⁻¹ of tomato extract (Semiarti et al., 2010). The optimal concentration of AS might be depends on the species of orchid or plant. Using 200 μM AS, Atichart et al. (2007) obtained 38% transformation efficiency in *D. Secundum*, but Venkada et al. (2007) got no-significant different of transformation frequency in rice. It might also depends on the strain of Agrobacterium used in the experiments. It is worth to try to add acetosyringone into Agrobacterium
culture prior to transformation and cocultivation as the same method treated for the black orchid in Phalanopsis amabilis and other orchids. The high frequency results will support both the conservation effort and commercial business of orchids.

Table 1. Frequency of genetic transformation of Hd3a gene into Black Orchid after Acetosyringone treatment

<table>
<thead>
<tr>
<th>No</th>
<th>AS concentration (mg.l(^{-1}))</th>
<th>Number of transformed protocorm</th>
<th>Resistant protocorm</th>
<th>Transformation frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>470</td>
<td>78</td>
<td>17.0</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>374</td>
<td>63</td>
<td>16.8</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>525</td>
<td>222</td>
<td>42.4</td>
</tr>
<tr>
<td>4</td>
<td>75*</td>
<td>273</td>
<td>134</td>
<td>49.1*</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>277</td>
<td>33</td>
<td>11.9</td>
</tr>
</tbody>
</table>

*Indicates the highest transformation frequency that reached after addition of 75 mg.l\(^{-1}\) AS in Agrobacterium culture prior to co-cultivation.
Fig 3. Selected protocorm after 2 weeks on selection medium contain 20 mg.l⁻¹ Hygromycin with various concentration of Acetosyringone when preculture and cocultivation. A: 0 mg.l⁻¹ AS, B: 25 mg.l⁻¹ AS, C: 50 mg.l⁻¹ AS, D: 75 mg.l⁻¹ AS, E: 100 mg.l⁻¹ AS

Fig 4. PCR analysis for Hd3a integration. A 100 bp amplified DNA fragment indicates Hd3a is integrated into transformat genom of Black Orchid. NT: Non Transformant, T1,T2: Transformant, M: lambda/StyI DNA Marker

CONCLUSION

Acetosyringone improves the frequency of genetic transformation of Hd3a gene of rice in black orchids with optimal concentration is (50-75) mg.l⁻¹ that increases the frequency for about 42-49%.

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REFERENCES


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