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REGULAR ARTICLE

Optimization of various factors affecting *Agrobacterium*-mediated transformation and regeneration of putative transgenic Malaysian MR219 rice with *Bacillus* SP 289 endo-β-1,3-1,4-glucanase gene

Zuraida Ab Rahman[1]*, Noriha Mat Amin[1], Mohd Yusof Nor Rahim[1], Azlinda Erny Yunus[1], Rohaiza Ahmad Redzuan[1], Muhamad Ridzuan Rashid[1], Asfaliza Ramli[2], Ayu Nazreena Othman[1], Marzukhi Hashim[1], Rahiniza Kamaruzaman[2], Sreeramanan Subramaniam[3]

[1]Biotechnology & Nanotechnology Research Centre, MARDI Headquarters, MARDI HQ, Persiaran MARDI-UPM, 43400 Serdang, Selangor, Malaysia, [2]Rice Industry Crop Centre, MARDI Headquarters, Research Centre, MARDI Headquarters, MARDI HQ, Persiaran MARDI-UPM, 43400 Serdang Selangor, Malaysia, [3]School of Biological Sciences, Universiti Sains Malaysia, Georgetown, 11800 Penang, Malaysia

ABSTRACT

Sheath blight disease caused by *Rhizoctonia solani* is the most destructive diseases in rice cultivation. Development of transgenic indica rice MR219 through *Agrobacterium tumefaciens* strain EHA 105 harbouring the plasmid pCAMBIA 1305.2 with *endo-beta-1,3-1,4-glucanase* gene from *Bacillus* SP 289 is one of the strategies to engineer disease resistance. Four optimisation parameters were examined such as *Agrobacterium* culture cell density (0.1 to 1.0 based on OD_{600nm}), callus immersion time in the *Agrobacterium* culture (30 to 120 minutes), duration of the subsequent drying time (15 to 120 minutes) and co-cultivation period (1 to 6 days). Hygromycin-resistant embryogenic calli developed after 8 to 10 weeks of transformation. Improved transformation rates were achieved when calli were incubated with an *Agrobacterium* suspension with a culture density of OD_{600nm} 0.2 for 30 mins, followed by 90 mins of drying time and co-cultivation for 3 days. PCR analysis of the transformants confirmed the presence of *Bacillus* SP 289 *endo-beta-1,3-1,4-glucanase* and *hpt* genes in the rice genome. The transgenic rice plants obtained in this study will be tested against sheath blight disease.

Keywords: Malaysian MR219; *Agrobacterium tumefaciens*; *Bacillus* SP 289; endo-beta-1,3-1,4-glucanase

INTRODUCTION

Rhizoctonia solani caused sheath blight disease in rice is one of the most widely distributed diseases globally. Crop losses may range from slight to heavy each year, depending on prevailing weather conditions, the stage of plant growth when infection occurs, the extent of infection, and the rice variety grown.

In recent years, resistant rice cultivars to *Rhizoctonia solani* have been developed by inserting chitinase genes into the rice plant genome. Previously, Datta et al. (2001) demonstrated production of fungal resistant rice cultivars with a rice chitinase gene (RC7) obtained in *R. solani*-infected rice plants. Similarly, Kumar et al. (2003) reported on the successful production of *R. solani*-resistant rice with different type of rice chitinase gene (chi11). In addition, higher number of research reports on the use of glucanase gene in transgenic research for the development of fungal resistant rice plants. Nishizawa et al. (2003) introduced β-1,3 and 1,4-glucanase gene (*Gns1*) in rice in order to increase fungal disease resistance. To attain an even higher degree of disease resistance, therefore multiple genes of fungal wall-degrading enzymes (chitinase, glucanase and thaumatin-like proteins) were co-expressed in transgenic rice. For example, Mei et al. (2004) have introduced an *ech42* gene encoding endochitinase, the *nag70* gene encoding exochitinase and the *gluc78* gene encoding glucanase into rice. Finally, transgenic rice plants displayed a superior resistance against sheath blight disease were successfully obtained.

***Corresponding author:**
Zuraida Ab Rahman, Biotechnology & Nanotechnology Research Centre, MARDI Headquarters, MARDI HQ, Persiaran MARDI-UPM, 43400 Serdang, Selangor, Malaysia. E-mail: azuraida@mardi.gov.my

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Transgenic plant technology provides an avenue for genetic manipulation of rice to enhance its agronomic performance by increasing resistance to biotic and abiotic stresses (Sahrawat et al., 2003). Even though various methods have been employed to introduce cloned DNA sequences into the rice plant, however *Agrobacterium tumefaciens*-mediated system is still preferable method since it possesses several advantages over other systems. These include the ability to transfer large segments of DNA with precise insertion of transgenes resulting in fewer copies of inserted genes at lower cost (Smith and Hood, 1995; Dai et al., 2001).

In the present work, an *endo-β-1,3-1,4-glucanase* gene has been introduced from locally isolated rhizosphere soil bacteria, *Bacillus* SP 289 into embryogenic calli of indica rice MR219 using *Agrobacterium*-mediated transformation system. The aim of this study was to produce MR219 rice plants against sheath blight disease. The effects of four experimental variables, namely *Agrobacterium* culture cell density, immersion time, duration of the subsequent drying time, and co-cultivation period were examined in order to optimise the transformation system.

MATERIALS AND METHODS

Plant materials and explants

Embryogenic calli used derived from the dough stage of rice seeds. The seeds were dehusked, cleaned and sterilized by immersion in 100% ethanol for 1-2 min, followed by immersion in 100% Clorox (sodium hypochlorite 5.25%) supplemented with a few drops of Tween-20 for 30 min. After rinsing few times with sterile distilled water, the seeds were introduced on callus induction Murashige and Skoog (MS) medium (Murashige and Skoog 1962) with 30 g/L sucrose, 10 mg/L glucose, 0.3% Gelrite agar, 10 mg/L naphthaleneacetic acid and 1 mg/L 2,4-D (Zuraida et al., 2010). Cultured seeds maintained in the dark condition at 25-27°C under cool. Six week-old embryogenic calli were used for the transformation of indica rice MR219.

Agrobacterium strain and plasmid

A. tumefaciens strain EHA 105 was used in genetic transformation. The bacterium harboured the plasmid pCAMBIA 1305.2 (http://www.cambia.org.au/) with the *endo-beta-1,3-1,4-glucanase* gene driven by the Cauliflower Mosaic Virus 35S promoter (CaMV35S) and nopaline synthase (NOS) terminator. This vector had hygromycin phosphotransferase (*hpt*) gene in the T- DNA region driven by a double-enhancer version of the CaMV35S promoter and terminated by the CaMV35S polyA signal which conferred resistance to the antibiotic hygromycin used as a transformation selection marker (Fig. 1).

Evaluation of experimental variables influencing transformation efficiency

Several factors affecting the *Agrobacterium*-mediated transformation frequency in MR219 callus were evaluated. The factors were such as *Agrobacterium* culture cell density (0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 OD_{600nm}), callus immersion time in *Agrobacterium* culture (30, 60, 90 and 120 minutes), subsequent duration of drying before co-cultivation (15, 30, 45, 60 and 90 minutes), and co-cultivation period (1, 2, 3, 4, 5 and 6 days).To determine the optimum conditions for transformation, one factor of the standard conditions was changed each time. The transformed calli were transferred to a pre-regeneration medium (Zuraida et al., 2010) supplemented with 100 mg/L carbenicillin, 100 mg/L cefotaxine and 50 mg/L hygromycin as a selective agent. Transformed calli were incubated in dark condition at 27 ± 1°C, and were then sub-cultured at 2-week intervals on the same medium for the selection process.

Regeneration of transgenic rice plants

The transformed whitish embryos were transferred to MS regeneration media (Zuraida et al. 2011) supplemented with 30 mg/L hygromycin B, 100 mg/L carbenicillin and 100 mg/L cefotaxine for further regeneration. The cultures were then kept at 26 ± 2 °C under 16-h photoperiod. Total number of regenerated plants was determined after 4 to 8 weeks. The percentage of plant regeneration was worked out as follows:

Plant regeneration response (%) = Total number of confirmed transformants callus/Total number of calluses x 100.

Putative regenerated plantlets with good shoot and root growth were taken out for hardening. The plantlets were

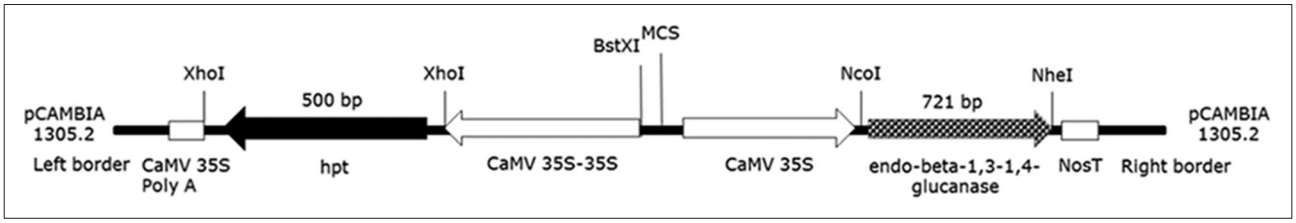

Fig 1. Schematic diagram of the plasmid pCAMBIA1305.2 and T-DNA construct.

washed repeatedly to remove adhering agar and kept in vermiculate for under culture conditions. Plantlets were then transferred into small plastic cups containing autoclaved mix soil and vermiculate (20:80) covered with plastic bags. For two weeks, plantlets were allowed to be under culture conditions. Polybags were removed and plantlets were transferred to mud pots then transferred to the transgenic glass house for further maturity.

PCR analysis of transgenic plants

To confirm the presence of the *endo-beta-1,3-1,4-glucanase* gene in transgenic rice plants, DNA was isolated from 0.5 g of fresh young leaves using DNeasy Plant Mini Kit (QIAGEN, Germany). For the PCR analysis, 400 ng of plant genomic DNA or 50 ng of plasmid DNA were used in 25 µL reaction mixure. The primers designed to amplify 720-bp fragments of *endo-beta-1,3-1,4-glucanase* gene at 55°C

(F 5'-CTAATTCTTGTCACTGGATTGTTTTTG-3'; R 5'GTATAGCGGATTTACACCATTGTAGG-3') and 508-bp fragments of *hpt* gene at 50°C (F 5'-ATGAAAAAGCCTGAACTCACCGCGA-3'; R 5'TCCATCACAGTTTGCCAGTGATACA-3').

Amplification was performed in a PTC-200™ Programmable Thermal Controller (MJ Research, Inc., USA). The PCR program profile for both genes was as follows; initial denaturation at 95 °C for 3 min, followed by 35 cycles of 94 °C for 30 sec, 45 sec at the annealing temperature of each gene and 1 min at 72 °C, with a final extension at 72 °C for 10 min. The amplified products were separated on 1 % agarose gel and visualized by staining with ethidium bromide.

Statistical analysis

Total of 100 embryogenic callus samples used in each experiment and were repeated two times. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's test to determine significant ($P \leq 0.05$) differences. P value of less than 0.05 was considered as significant.

RESULTS AND DISCUSSION

Using an appropriate *Agrobacterium* cell density is critical to successful genetic transformation. An optimal density 0.1 - 0.2 was found to be optimal for the survival of the explants after immersion in the bacterium medium for 30 minutes. Increasing bacterium concentrations (> 0.2 OD_{600nm}) resulted in a significant decrease in the number of hygromycin-resistant embryogenic calli obtained (Fig. 2).

Hg-resistant embryogenic calli were obtained between eight and ten weeks after selection process (Fig. 3 a,b). Moreover,

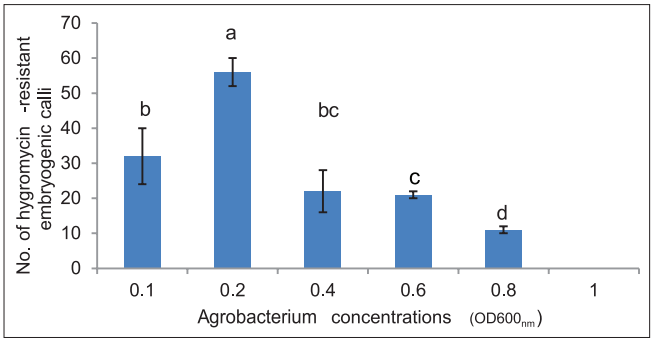

Fig 2. Effect of *Agrobacterium* culture cell density ($OD_{600\ nm}$) on the production hygromycin-resistant embryogenic calli of indica rice.

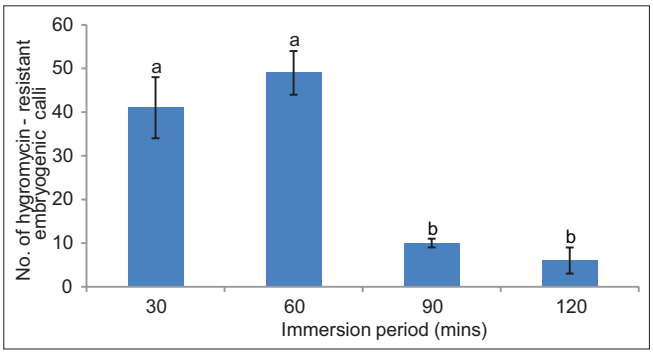

Fig 3. Effect of immersion time on the production hygromycin-resistant embryogenic calli after transformation of indica rice.

no transformed calli were obtained when bacterium cell densities used at 1.0 OD_{600nm} and untransformed calli (Fig. 3c). This result was contrast to Sreeramanan et al. (2009), who reported that low transformation efficiency with *Agrobacterium* at a cell density of 0.2 at OD_{600nm} was attributed due to lack of sufficient *Agrobacterium* cells to transfer T-DNA into orchid cells. According to Zuraida et al. (2011), delivery of *gusA* gene was most efficient in rice cultivar MR232 by using *Agrobacterium* cell density of OD_{600nm} 0.6. Yong et al. (2006) reported that the highest rate of genetic transformation of members of the Melastomataceae family with *Agrobacterium* LBA4404 was obtained at a cell density of 0.8 OD_{600nm} ($1x10^7$cfu ml^{-1}). They also found that higher concentrations decreased transformation efficiency for shoot and node explants. With Vanda Kasem's Delight (VKD) orchid, *Agrobacterium* suspension culture at 0.8 OD_{600nm} produced highest production (91.6%) of GUS positive explants, followed by 0.6 OD_{600nm} (60%) and 0.4 OD_{600nm} (51.6%) (Pavallekoodi et al., 2014).

Differences in the production hygromycin-resistant embryogenic calli after transformation event were observed for each level of bacterial density. Calli immersed for 60 min in *Agrobacterium* culture (0.2 OD_{600}) produced the most hygromycin-resistant embryogenic calli (Fig. 4). This immersion or infection period allowed sufficient time for

the infection process leading to transformation in the calli, whereas longer immersion periods were not helpful. Pavallekoodi et al. (2014) indicated that a 30-minute immersion period in *Agrobacterium* culture was the optimum for transforming *Vanda* orchids, with immersion durations of 10 to 20 min being less effective. According to Orlikowska et al. (1995), increased bacterial infectivity at longer duration time may lead to hypersensitive response and causes reduction of regeneration frequency.

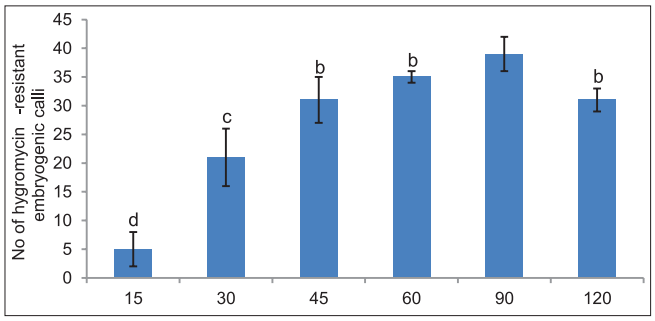

Fig 4. Effect of the drying period following infection (min) on the production hygromycin-resistant embryogenic calli of indica rice.

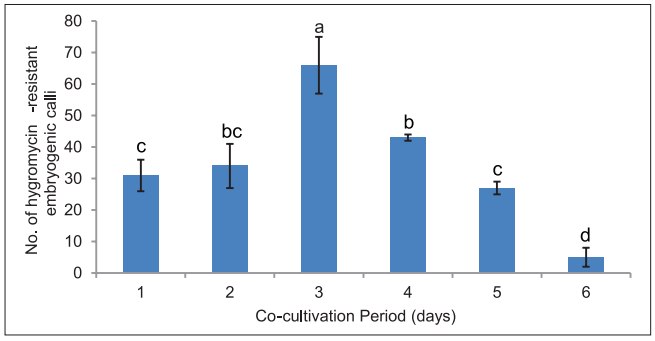

Fig 5. Effect of co-cultivation periods on the production hygromycin-resistant embryogenic calli of indica rice.

The effect of the drying period of calli after infected with *Agrobacterium* is summarized in Fig. 5. The highest number of hygromycin-resistant embryogenic calli was obtained with calli drying for 90 minutes followed by 60 minutes on sterile filter paper prior to co-cultivation. *A. tumefaciens* required specific time period for attachment and maximum transfer of T-DNA to the explants. Even though shorter infection period would produce low numbers of transformed explants, but an excessive period resulted hypertonic conditions in the cells that causes to rupture. Alternatively, hyper-activation of defense mechanisms induced in the cells could lead to lethality (Mannan et al., 2009). A co-cultivation incubation period of 3 days was found suitable for the transfer of TDNA into the indica rice calli (Fig. 6).These results are in accordance with Mannan et al. (2009), who reported that co-cultivation with *Agrobacterium* for a period of 3 to 4 days gave maximum positive GUS expression in *Artemisia absinthium* regenerated plantlets.

The duration of co-cultivation of cell cultures with *Agrobacterium* had an important influence on transformation efficiency. In this study, the co-cultivation period of the rice calli with *Agrobacterium* was optimal at 3 days. In comparison, the number of *Arabidopsis* transformed calli increased with co-cultivation time, reaching an optimum

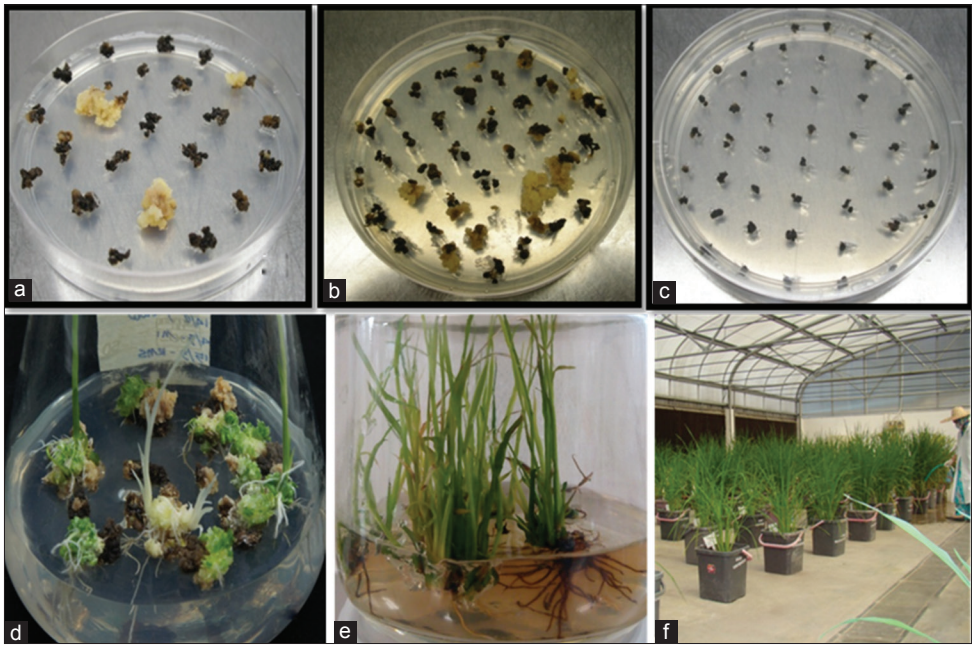

Fig 6. Production of transgenic MR219 rice plants with *Bacillus* SP 289 *endo-beta-1,3-1,4-glucanasegene* using *Agrobacterium*-mediated transformation. Development of hygromycin-resistant embryogenic calli (a and b) after eight to ten weeks of transformation. Untransformed control calli did not show further development (c). Regenerated shoots (d) and emerging plantlets (e), plant in transgenic glass house (f).

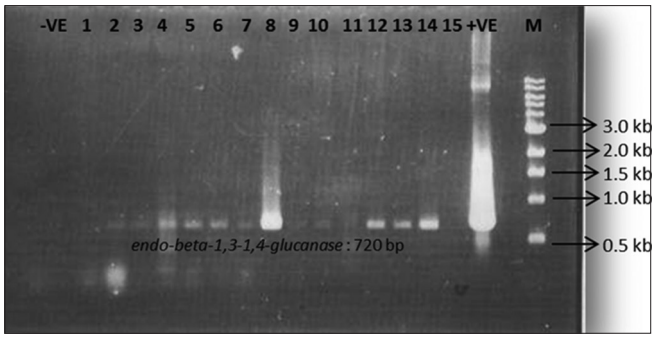

Fig 7. PCR amplification of *endo-beta-1,3-1,4-glucanase* gene from transgenic R_0 lines transformed with the gene construct; -VE: Negative control, Lane 1-15: Transgenic lines 1-15, +VE: Positive control, M: 1kb DNA Ladder (New England Biolabs, USA)

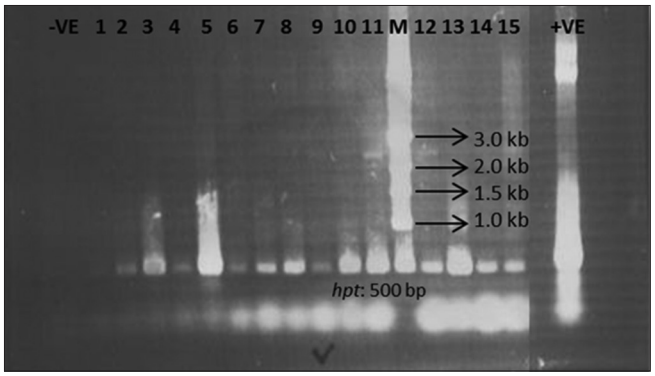

Fig 8. PCR amplification of *hygromycin phosphotransferase* gene (*hpt*) from transgenic R_0 lines transformed with the construct; -VE: Negative control, Lane 1-15: Transgenic lines 1-15, +VE: Positive control, M: 1kb DNA Ladder (New England Biolabs, USA)

after 48 hours and longer co-cultivation time eventually reduced viability of the plant cells and delay in the growth of transformed explants (Christoph et al., 1997). Similarly, Vergauwe et al. (1998) obtained the best results after co-cultivating *Artemisia annua* with *Agrobacterium* for 2 to 3 days. They also noticed that a longer co-cultivation period did not improved transformation efficiency.

Transformed somatic embryos proliferate more actively and have minimal browning (Fig. 3d). Regenerated shoots with root were then transferred into free-hg media (Fig. 3e) before brought to the transgenic glasshouse for further development (Fig. 3f). Integration of transgenes in the genome of putative transformants was determined by PCR analysis of the R_0 rice plants. Amplified product of 720 bp DNA fragment for *endo-beta-1,3-1,4-glucanase* gene was obtained from 12 lines and a 500 bp DNA fragment for *hpt* gene was obtained for 14 lines of the MR 219 rice plants, respectively (Figs. 7 and 8).

Hence, these findings suggest that the *Bacillus* SP 289 *endo-beta-1,3-1,4-glucanase* gene could be utilized as a genetic source of disease resistance against sheath blight.

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Author contributions

Zuraida Ab Rahman: designed and carried out the experiments, provided the materials and ideas for the study. Wrote the article; Noriha Mat Amin: Conducted field analysis, designed and carried out the experiments, Wrote the article; Mohd Yusof Nor Rahim: Conducted field analysis; Azlinda Erny Yunus: Conducted field analysis. Rohaiza Ahmad Redzuan: Conducted field analysis; Muhamad Ridzuan Rashid: Conducted field analysis; Asfaliza Ramli : provided the materials and ideas for the study. Ayu Nazreena Othman: designed and carried out the experiments, Marzukhi Hashim: provided the materials and ideas for the study. Rahiniza Kamaruzaman: provided the materials and ideas for the study. Sreeramanan Subramaniam: Wrote the article

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