INTRODUCTION OF THE SERINE *GREEN FLUORESCENT PROTEIN (SGFP)* GENE INTO *Pyricularia grisea* RACE DC4 ISOLATED FROM *Digitaria ciliaris* USING *Agrobacterium tumefaciens*-MEDIATED GENETIC TRANSFORMATION

STEPHANIE[1], UTUT WIDYASTUTI[2,3*] and SURYO WIYONO[4]

[1]*Program Study of Biotechnology,Graduate School, Institut Pertanian Bogor; Bogor 16680, Indonesia*
[2]*Research Center for Bioresources and Biotechnology, Institut Pertanian Bogor; Bogor 16680, Indonesia*
[3]*Department of Biology, Faculty of Mathematics and Natural Sciences, Institut Pertanian Bogor; Bogor 16680, Indonesia*
[4]*Department of Plant Protection, Faculty of Agriculture, Institut Pertanian Bogor, Bogor 16680, Indonesia*

Received 24 July 2013/Accepted 12 September 2014

ABSTRACT

Blast disease (caused by *Pyricularia grisea*) has long been known as a serious problem for upland rice. Now, it also attacks lowland rice. However, the mechanism facilitating this range expansion is still unknown. One option is to insert a marker into *P. grisea* so that it can be used to monitor the spread of *P. grisea* infection. *sGFP* (*Serin Green Fluorescent Protein*) gene has been used to monitor gene expression of specific tagged proteins for the study of fungal cell. In this study, the *sGFP* gene has been integrated into the genome of *P. grisea* dc 4 from *Digitaria ciliaris*. The plasmid *sGFP* was introduced into *A. tumefaciens* using the triparental mating method (TPM). Genetic transformation was performed by co-cultivating spores of *P. grisea* dc4 with *A. tumefaciens* LBA4404–pCAMB-s*GFP*. The *P. grisea* dc4 transformant was selected by using a selection medium containing 300 µg/mL of hygromycin. The integration of the *sGFP* gene into the genome was confirmed by PCR using the *sGFP*'s specific primer pair, the *sGFP*-Nos terminator primer pair and the β-Tubulin primer pair as an internal control. Expression of the *sGFP* gene from the *P. grisea* dc4 transformant was detected with blue light fluorescent microscopy.

Keywords: *Agrobacterium tumefaciens*-mediated transformation, *Digitaria ciliaris*, *Pyricularia grisea* dc4, *Serine Green Fluorescent Protein*

INTRODUCTION

Pyricularia grisea (teleomorph *Magnaporthe grisea*), synonymous with *Pyricularia oryzae* Cav, is a rice plant pathogen in many countries and is known as the agent causing blast disease (Rho *et al.* 2001). The disease is estimated to be responsible for 30% of annual yield loss, the equivalent in food volume to meet the needs of 60 million people (Dagdas *et al*. 2012). In Indonesia, blast infestation reached 36,727 ha of the total 13,153,080 ha area of rice cultivation in 2012 (Ministry of Agriculture 2013). Blast disease has been known as a serious problem for upland rice, however, recent results showed that the blast pathogen also attacks rice planted in lowlands or irrigated land (Sobir *et al*. 2003). To date, the mechanism of blast disease transmission from upland rice to lowland land has not been examined.

Pyricularia grisea is a pathogen on more than 50 species of wild grass in the vicinity of rice field (Couch & Khon 2002). *P. grisea* has a wide variety of hosts besides rice plant; among them are *Triticum aestivum*, *Zea mays*, *Eleusine coracana* (finger millet), *Setaria italica* (cultivated grass), *Brachiaria mutica* (Couch & Khon 2002; Listiyowati *et al*. 2011). Because of this wide host range, cereal grasses and wild grasses around the area of cultivation are also prone to *P. grisea* infection and therefore, could become alternative hosts as well as alternative inocula sources for spreading disease.

* Corresponding author : ututsuharsono2002@ yahoo.com

Research showed that grasses growing around rice field could become temporary hosts for fungi that cause blast disease (Listiyowati *et al*. 2011). Those fungi were capable of infecting rice plants and showed some genetic structure alterations from the original grass isolate. *P. grisea* isolated from *D. ciliaris* grass (dc4) is able to infect rice plants that are moderately resistant and susceptible plants. This isolate of dc4 that switches host undergoes genetic alterations, which are marked by changes in SCAR marker for Cut1, PWL2, Erg2 as well as physiological race based on the ability to infect differential rice varieties in Indonesia (Listiyowati *et al*. 2011). This is presumed as a genomic adaptation response of the fungi isolate to the new host. However, the mechanism that causes such change is still unknown. Therefore, as an initial step it is necessary to insert a marker into *P. grisea* to monitor *P. grisea* infection.

According to Lorang *et al.* (2001), the *GFP* gene could be used as a tool in studying interactions between fungi and plants. *GFP* has also been used to monitor the virulence of blast-causing fungi. *GFP* gene has several advantages as a marker. *GFP* gene detection does not require any substrate addition to obtain the visualization; it does not require special treatment of the tissue and its presence in the cell is not harmful to the cell itself. These advantages make *GFP* a great marker in gene transformation and expression (Lorang *et al*. 2001). Today, the *GFP* gene has been shown to be expressed in 16 species of 12 genera of fungi including *M. grisea* (Lorang *et al*. 2001). The *GFP* gene has been successfully used as important marker for several fungi that cause diseases and *P. grisea* is among them (Balhadere & Talbot 2001).

sGFP gene transfer into fungi genome requires a biological vector. *Agrobacterium tumefaciens* has been long used as a biological vector for gene transfer in plants. Besides transferring genes to its host plant, *A. tumefaciens* is also able to transfer DNA to yeasts and filamentous fungi (Combier 2003). The objective of this research was to introduce *sGFP* gene into *P. grisea* dc4 isolated from *D. ciliaris* using *A. tumefaciens*-mediated genetic transformation. Subsequently, there would be a *sGFP* gene introduction into the fungi genome. The success of this gene introduction could be developed to facilitate molecular studies of pathogen-host interactions.

MATERIALS AND METHODS

Triparental Mating

The introduction of binary plasmids that contain *sGFP* gene into *A. tumefaciens* was done with the TPM assay described by Hanum (2011). Three bacteria: *E. coli* DH10B donor containing pCAMB-*sGFP* plasmids, *E. coli* DH1 containing helper pRK2013 plasmids and *A. tumefaciens* LBA4404 as the recipient strain were used in TPM assay. The three bacteria were grown in solid LB to prevent conjugation. *A. tumefaciens sGFP* transformants were then identified using colony PCR with specific primers *sGFP*-F and *sGFP*-R.

Fungal Strains and Culture Conditions

P. grisea race dc4 isolated from *D. ciliaris* was provided by Mycology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Institut Pertanian Bogor. Fungal cultures were grown on oatmeal agar medium (oma: 30 g of oatmeal for 1 L). Fungi were grown in OA medium and incubated for 7-10 days at 28 °C. For production of conidia, fungal cultures were grown under light of a near-UV lamp for 5 to 6 days using sterilized water (Munandhar *et al.* 1998).

Transformation

One colony of *A. tumefaciens* containing pCAMB-*sGFP* plasmids was grown on 2 mL of Minimal Medium (Hooykaas *et al.* 1979) supplemented with 100 µg/mL streptomycin, 60 µg/mL kanamycin, and 50 µg/mL hygromycin at 28 °C for 48 hours with 250 rpm rotation in no light condition. The bacterial culture was diluted with 5 mL Induction Medium (IM) containing 200 µM acetosyringone to an optical density of 0.15 at A600. The bacterial culture of *A. tumefaciens-sGFP* was grown for an additional 4-6 hours at 28 °C with 250 rpm rotation to reach an A600 of 0.5 (Betts *et al*. 2007). Approximately 100 µl of *P. grisea* dc4 spores (10^6 spores/mL) was mixed with 100 µL of *A. tumefaciens-sGFP* cells (A600 = 0.5) then added with acetosyringone 200 µM, and incubated for 30 minutes with no light. The co-cultivated cultures were then plated into IM medium and incubated at 28 °C for 48 hours. The co-cultivation result was transferred into Complete Medium containing 300 µL/mL

hygromycin and 200 µg/mL cefotaxime and incubated at 28 °C for 5-7 days. Single spore selection was done by spreading 100 µL conidia suspension on OA medium that contained 300 µg/mL hygromycin and incubated for 5-7 days until *P. grisea* dc4 single spores appeared (Rho *et al*. 2001 modified by Betts *et al*. 2007).

Analysis of Transformant

Genomic DNA of *P. grisea* mycelium from transformant and non-transformant *P. grisea* were isolated for verification using PCR. DNA isolation was done with the method described by Listiyowati *et al*. (2011) using 2% *cetyltrimethyl ammonium bromide* (CTAB). The isolated DNA *sGFP* gene was then amplified through PCR using *sGFP*-F and *sGFP*-R primers as well as combined primers of *sGFP*-F and Nos-R. The PCR mixture used consisted of 1 µL (100 ng) genomic DNA, 0.5 mM forward primers, 0.5 mM reverse primers, 5 µL PCR mix (Fermentas) and added with ddH_2O up to 10 µL of total volume. The PCR program to amplify *sGFP* fragments consisted of: pre-denaturation at 94 °C for 1 minute, denaturation at 94 °C for 1 minute, annealing at 53 °C for 30 seconds, elongation at 72 °C for 1 minute and final elongation at 72 °C for 5 minutes; this process was run in 35 cycles. The PCR results were visualized through electrophoresis on 1% (b/v) agarose gel at 100 volt for 30 minutes in TAE 1 and continued with gel immersion in 0.5 mg/L EtBr for 20 minutes and were visualized under UV transluminator (Shanti 2008).

Microscopy

To visualize *P. grisea* transformants, they were subcultured in OA medium containing 300 µg/mL hygromycin. The culture was incubated at room temperature for 6 days. The transformants were grown under the light of a near-UV lamp for 5 to 6 days. The acquired spores were then observed using an Olympus BH2-RFCH microscope on the fluorescent setting with a 515 bandpass (blue light) emission fillter.

RESULTS AND DISCUSSION

Triparental Mating (TPM)

The plasmid pCAMB-*sGFP* was introduced into *A. tumefaciens* using the TPM method (Fig. 1). Plasmids contained in *E. coli* DH10B were moved into *A. tumefaciens* LBA4404 through conjugation with the help of *E. coli* pRK2013. Triparental mating generated several colonies growing in a medium containing 50 µg/mL kanamycin, 50 µg/mL streptomycin and 50 µg/mL hygromycin. The only bacterium able to grow in that selective medium was *A. tumefaciens* LBA4404 containing pCAMB-*sGFP* plasmids. Colonies obtained from TPM that were able to grow in the selective medium were then analyzed with PCR.

The results of the PCR analysis showed that colonies of *A. tumefaciens* with *sGFP*-F and *sGFP*-R primers produced amplicon with the size of 643 bp (Fig. 1). The results of this amplification had the same size as the positive control (Fig. 1). This showed that pCAMB-*sGFP* plasmid was successfully introduced into *A. tumefaciens* through the TPM method. This method was previously used by Hanum (2011) to move the binary plasmid pMSH-*MmCuZn-SOD* into *A. tumefaciens*. According to Wise *et al.* (2006) TPM is a quite efficient method for transferring gene in a non-conjugated plasmid into *A. tumefaciens*.

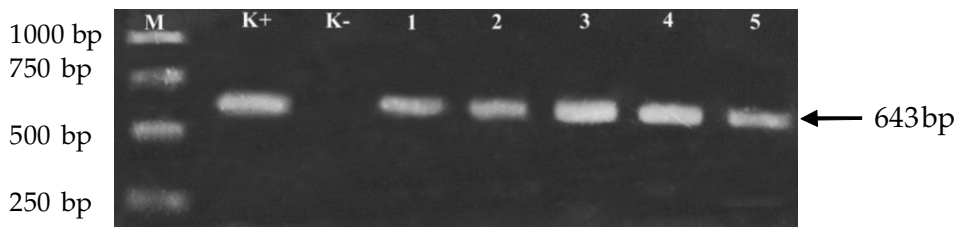

Figure 1. PCR identification of the *sGFP* gene introduced into *A. tumefaciens* LBA4404 colonies using TPM. M: marker 1 kb; K+: Plasmid pCAMB-*sGFP* (positive control); K-: ddH_2O (negative control); 1-5: *A. tumefaciens* transformant *sGFP*

Transformation

Genetic transformation of *P. grisea* dc4 using *A. tumefaciens* was achieved using the method described in Rho *et al.* (2001) and Betts *et al.* (2007). One hundred microliter (100 µL) of spores (10^6 per mL) were co-cultivated with 100 µL of bacterial cells in an induction medium that had been added to 200 µM acetosyringone for 48 hours. An acetosyringone concentration of 200 µM is important in fungal transformation to induce virA genes of *A. tumefaciens* so that T-DNA transfer may take place (Knight *et al.* 2009). An acetosyringone concentration of 200 µM is commonly used in fungal transformation with *A. tumefaciens* as a biological vector and it has been shown to have consistent results (Covert *et al.* 2001; dos Reis *et al.* 2004; Knight *et al.* 2009; Xiaoran *et al.* 2012).

P. grisea dc4 grown in hygromycin and cefotaxime selective medium was then cultured to produce spores. The spores were then spread in OA medium containing 300 µg/mL hygromycin and incubated for 5-7 days until some single spores that were transformed were obtained (Fig. 2). Selection of single spores was done to eliminate false transformant fungi (Betts *et al.* 2007). Mycelium growth on hygromycin medium indicated the success of transformation (Tucker & Orbach 2007). Some single spores that were not able to grow on hygromycin medium because the genes having important roles in growth might undergo some damage or their activities might be interrupted by the presence of foreign genes.

Single spores of *P. grisea* dc4 transformant and *P. grisea* dc4 non-transformant were cultured in a medium containing 300 µg/mL hygromycin then the growth diameter was observed at day 7, 14, and 21. The growth of *P. grisea* transformant increased rapidly which was 14.8 mm at day 7 became 28.7 mm at day 14 and became 37.3 mm at day 21. *P. grisea* non-transformant growth was slower which was 6.5 mm at day 7, 12.55 mm at day 14 and 17.3 mm at day 21 (Fig. 3). Hygromycin added into the OA medium was able to inhibit the growth of *P. grisea* non-transformants compared to *P. grisea* dc4 transformant *sGFP* (Fig. 4). This was caused by the inhibiting activity of hygromycin on the *P. grisea* dc4 non-transformant. The use of an appropriate selection marker is essential for the success of transformation. Selection using an antibiotic is performed to eliminate non-transformant cells as well as to ensure the resistance level carried by the transformant (Frandsen 2011). In this research *P. grisea* dc4 transformants *sGFP* were resistant to hygromycin because of the addition of the hygromycin-resistance gene contained in the pCAMB-*sGFP* plasmid. Betts *et al.* (2007) used the concentration of 300 µg/mL for the selection of both *M. grisea* transformant and non-transformant. In contrast, Shanti (2008) used a concentration of 225 µg/mL hygromycin to inhibit *P. grisea* 173 (originated from rice plant) non-transformant and transformant generated through speroplas. The difference in growth rate between the transformant on hygromycin medium and non-hygromycin medium (Fig. 4) was likely due to the influence of the number of gene copies integrated, however, further evidence for this is required.

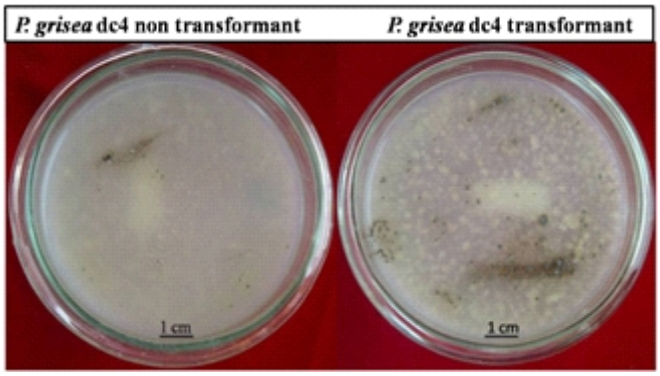

Figure 2. Single spore of *P. grisea* race dc4 at day 5 grown in OA medium containing 300 µg/mL hygromycin

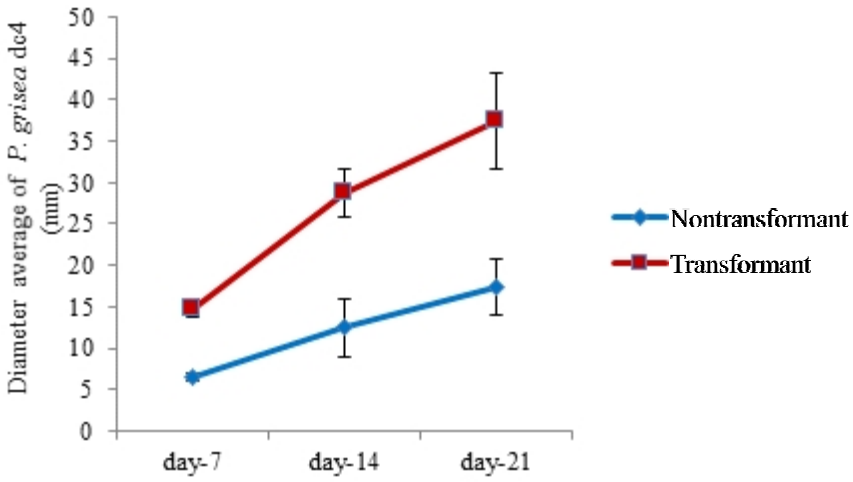

Figure 3. Average growth rate of *P. grisea* dc4 non-transformant and *sGFP* transformant in OA selective medium containing 300 µg/mL hygromycin

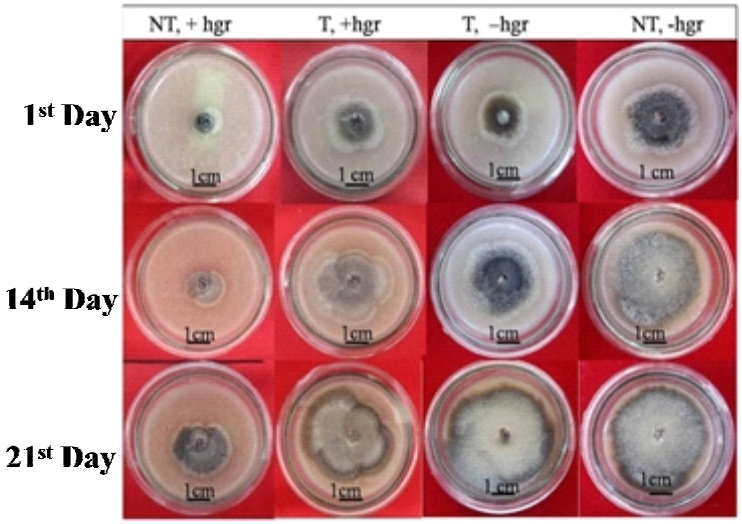

Figure 4. Growth of *P. grisea* dc4 non-transformant (NT) and transformant (T) in OA medium containing 300 µg/mL hygromycin (hgr+) and without hygromycin (hgr-)

Analysis of Transformant

Putative transgenic *P. grisea* dc4 from grass that had been selected in hygromycin medium was then analyzed using PCR with specific primers *sGFP*-F and *sGFP*-R as well as *sGFP*-F and Nos-R primers to detect the presence of *sGFP* gene. PCR molecular analysis generated amplicons of 643 bp and 899 bp which were in alignment with the positive control amplicon (Fig. 5). PCR of non-transformant DNA did not generate *sGFP* gene amplicons. This result showed that the *sGFP* gene was inserted into the fungal genome. The *B-tubulin* gene was used as an internal control to ensure the amplified DNA was in good condition. The *B-tubulin* PCR using Bt1aF and Bt1aR primers generated amplicons of 550 bp (Fig. 5).

P. grisea dc4 with *sGFP* gene insertion was the bright green fluorescence observed using a fluorescence microscope (Fig. 6). This showed that the *sGFP* gene was well integrated in the fungal genome and constitutively expressed in *P. grisea* dc4. The use of *sGFP* requires a promoter which is needed for *sGFP* expression. The pCAMB-*sGFP* plasmid receiving *sGFP* gene insertion from the pCT74 plasmid was able to express the *sGFP* gene under the control of the ToxA promoter (Lorang *et al.* 2001).

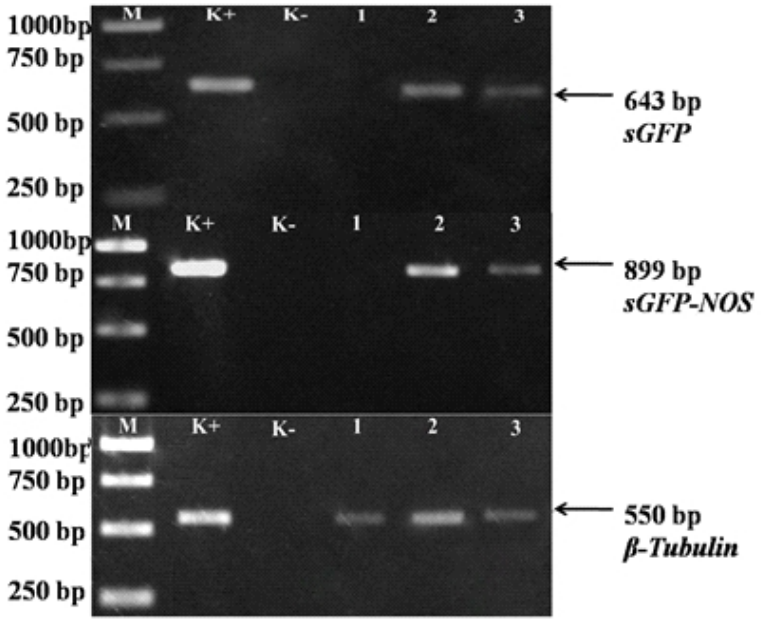

Figure 5. PCR results of *P.grisea* dc4 from transformation. M: Marker 1 kb; K+ *sGFP* dan *sGFP-Nos*: plasmid pCAMB-*sGFP*, K+ *β-Tubulin*: *P. grisea* ras 173 (positive control); K-: ddH2O (negative control); 1: DNA *P. grisea* dc4 nontransformant; 2-3: DNA *P. grisea* dc4 transformant *sGFP*

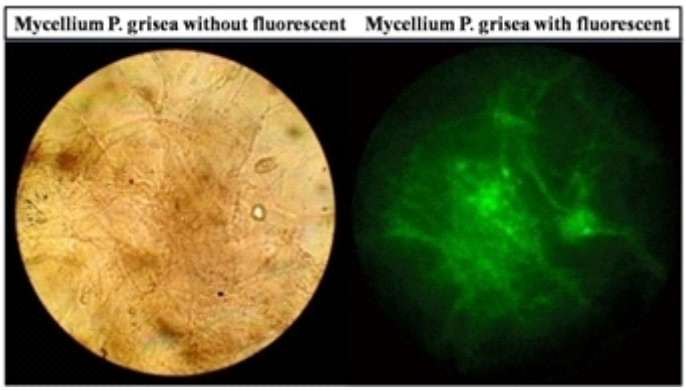

Figure 6. *P. grisea* dc4 *sGFP* transformant mycelium 7 hours after spore harvest observed under microscope without and with fluorescent

CONCLUSIONS

Pathogenic *P. grisea* dc4 from *D. ciliaris* had been successfully transformed with the *sGFP* gene using *A. tumefaciens*-mediated genetic transformation. The *sGFP* gene had been integrated into the *P. grisea* genome. Mycelia fluorescence had been observed under a fluorescence microscope.

ACKNOWLEDGEMENTS

Thanks to Dr. Osbourn from John Innes Institute, England who provided plasmid pCAMB-*sGFP*. Thanks to Margaret Cargill and Pattric O'Connor for early reading this manuscript. This research was funded by I-MHERE B2C titled "Biological Role of Rice Blast Disease to Develop Rice Plant Tolerance to Rice Blast" under the name of Dr Utut Widyastuti, agreement letter No : 12-IT3.24.4/SPP-I-MHERE/2012.

REFERENCES

Balhadere PV, Talbot NJ. 2001. PDE1 encodes a P-type ATPase involved in appressorium-mediated plant

infection by the rice blast fungus *Magnaporthe oryzae*. Science Direct 44:1035-49.

Betts MF, Tucker SL, Galadima N, Meng Y, Patel G, Li L, Donofrio N, Floyd A, Nolin S, Brown D *et al*. 2007. Development of a high throughput transformation system for insertional mutagenesis in *Magnaporthe oryzae*. Science Direct. 44:1035-49.

Combier JP, Melayah D, Raffier C, Gay G, Marmeisse R. 2003. *Agrobacterium tumefaciens*-mediated transformation as a tool for insertional mutagenesis in the symbiotic ectomycorhizal fungus *Hebeloma cylindrosporum*. Microbiol Lett 220:141-48.

Couch BC, Khon LM. 2002. A multilocus gene genealogy concordant with host preference indicates segregation of a new species, *Magnaporthe oryzae*, from *M. grisea*. Mycologia 94(4):683-93.

Couch BC, Fudal I, Lebrun MH, Tharreau D, Valent B, Van Kim P, Notteghem JL, Kohn LM. 2005. Origins of host-specific populations of the blast pathogen *Magnaporthe oryzea* in crop domestication with subsequent expansion of pandemic clones on rice and weeds of rice. Genetics 170:613-30.

Covert SF, Kapoor P, Lee MH, Goh J, Yoo SY, Park J, Jung K, Kim H, Park SY, Rho HS *et al*. 2001. *Agrobacterium tumefaciens*-mediated transformation of *Fusarium circinatum*. Myco Res 105:703-9.

dos Reis MC, Pelegrinelli Fungaro MH, Delgado Duarte RT, Furlaneto L, Furlaneto MC. 2004. *Agrobacterium tumefaciens*-mediated genetic transformation of the entomopathogenic fungus *Beauveria bassiana*. J Microbiol Methods 58:197-202.

Dagdas YF, Yoshino K, Dagdas G, Ryder LS, Bielska E, Steinberg G, Talbot NJ. 2012. Septin-mediated plant cell invasion by the rice blast fungus *Magnaporthe grisea*. Science. 336:1590-5.

Departemen Pertanian, Direktorat Jenderal Tanaman Pangan. 2012. *Evaluasi Prakiraan Serangan Utama OPT Utama Tanaman Padi, Jagung, Kedelai MT 2011/2012*. Jakarta (ID): Deptan.

Frandsen RJN. 2011. A guide to binary vectors and transformant for targeted genome modification in fungi using *Agrobacterium tumefaciens*-mediated transformation. J Microbiol Methods 87:247-62.

Hanum S. 2011. Isolasi pengklonan dan analisis ekspresi gen penyandi copper-zinc superoxide dismutase (cuZn-SOD) dari *Melastoma malabathricum*. Disertasi. Bogor (ID): Institut Pertanian Bogor.

Hao X, Ji Y, Liu S, Bi J, Bi Q, Pan J, Zhu X. 2012. Optimized integration of T-DNA in the taxol-producing fungus *Pestaliopsis maliocola*. African J Biotech 11(4):771-6.

Hooykaas PJJ, Roobol C, Schilperroot RA. 1978. Regulation of the transfer of Ti-plasmid of *Agrobacterium tumefaciens*. Microbiol 110:99-109.

Knight CJ, Bailey AM, Foster GD. 2009. *Agrobacterium*-mediated transformation of the plant pathogenic fungus *Verticillium albo-atrum*. Plant Pathol 91(3):745-50.

Listiyowati S, Utut W, Rahayu G, Hartana A, Jusuf M. 2011. Diversity of SCAR markers of *Pyricularia grisea* raced from *Digitaria ciliaris* following cross infection to rice. Microbiol Indones 5(1):1-8.

Lorang JM, Tuori RP, Martinez JP, Sawyer TL, Redman RS, Rollins JA, Wolpert TJ, Johnson KB, Rodriguez RJ, Dickman MB *et al*. 2001. Green fluorescent protein is lighting up fungal biology. Appl Environ Microbiol 67:1987-94.

Munandhar HK, Jorgensen HJL, Smedegaard-Petersen V, Mathur SB. 1998. Seedborne infection of rice by *Pyricularia grisea* and it's transmission to seedling. Plant Dis 82:1093-99.

Olmedo-Monfil V, Cortes-Penagos C, Herrera-Estrella A. 2004. Three decades of fungal transformation. Balbas P, Lorence A, editor. *Recombinant Gene Expression. Reviews and Protocols Volume 267*: *Methods in Molecular Biology*. Totowa (US): Human Press. p 297-309.

Rho HS, Kang S, Lee YH. 2001. *Agrobacterium tumefaciens*-mediated transformation of the plant pathogenic fungus, *Magnaporthe grisea*. Mol Cells 12:407-11.

Sesma A, Osbourn AE. 2004. The rice leaf blast pathogen undergoes developmental processes typical of root-infecting fungi. Nature. 431:582-86.

Shanti NR. 2008. Introduksi gen *serin-GFP* (*sGFP)* ke dalam genom *Pyricularia grisea*. Skripsi. Bogor (ID): Institut Pertanian Bogor.

Sobir, Andrianyta H, Amir M. 2003. SCAR (Sequence Characterized Amplified Region) analysis for blast resistant evaluation on 12 genotypes of rice. Bul Agron 31(1):21-5.

Tucker SL, Orbach MJ. 2007. *Agrobacterium*-mediated transformation to create an insertion library in *Magnaporthe grisea*. Ronald PC, Lorence A, editor. *Plant-Pathogens Interaction Methods and Protocols; Methods and Protocols Volume 354*: *Methods in Molecular Biology*. Totowa (US): Human Press. p 57-67.

Wise AA, Liu ZZ, Binns AW. 2006. Three methods for introduction of foreign DNA into *Agrobacterium*. Methods in Mol Biol 43-54.