Detection of Oil Palm Root Penetration by *Agrobacterium*-Mediated Transformed *Ganoderma boninense*, Expressing Green Fluorescent Protein

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Accepted for publication 27 November 2016.

ABSTRACT

A highly efficient and reproducible *Agrobacterium*-mediated transformation protocol for *Ganoderma boninense* was developed to facilitate observation of the early stage infection of basal stem rot (BSR). The method was proven amenable to different explants (basidiospore, protoplast, and mycelium) of *G. boninense*. The transformation efficiency was highest (62%) under a treatment combination of protoplast explant and *Agrobacterium* strain LBA4404, with successful expression of an *hyg* marker gene and *gus-gfp* fusion gene under the control of heterologous p416 glyceraldehyde 3-phosphate dehydrogenase promoter. Optimal transformation conditions included a 1:100 *Agrobacterium*/explant ratio, induction of *Agrobacterium* virulence genes in the presence

of 250 µM acetosyringone, co-cultivation at 22°C for 2 days on nitrocellulose membrane overlaid on an induction medium, and regeneration of transformants on potato glucose agar prepared with 0.6 M sucrose and 20 mM phosphate buffer. Evaluated transformants were able to infect root tissues of oil palm plantlets with needle-like microhyphae during the penetration event. The availability of this model pathogen system for BSR may lead to a better understanding of the pathogenicity factors associated with *G. boninense* penetration into oil palm roots.

Additional keywords: Basidiomycetes.

Ganoderma boninense is the primary agent of basal stem rot (BSR) disease (Ho and Nawawi 1985; Thompson 1931; Utomo et al. 2005) and has been continuously affecting the oil palm industry over the years. In mature palm trees, the fungus is able to infect up to 80% of the stand (Turner and Gillbanks 2003), limiting productive lifespan of the crop. Several *G. boninense* pathogenesis studies have improved the understanding of BSR development. Paterson (2007) demonstrated *G. boninense* infection via direct root-pathogen contact only. The soilborne *G. boninense* uses the root system to establish along the palm stem base, evident with visible formation of fruiting bodies (Ariffin et al. 2000; Sundram et al. 2011). In addition, microscopic examination of infected palm at the cellular level by Rees et al. (2009) revealed acquisition of biotrophic nutrition by the pathogen during colonization. *G. boninense* degrades lignin to weaken the rigid plant cell wall structure prior to starch consumption. The same study described *G. boninense* as a hemibiotroph, because the pathogen is able to switch from biotrophic nutrition (asymptomatic host) into necrotroph nutrition (symptomatic host) during the later stages of disease development. Generally, there are three key events held by hemibiotrophs during host colonization: (i) penetration, (ii) nutrient absorption at the expense of host, and (iii) host death (Rees et al. 2009; Turner 1981). Although the second and third components are well characterized in *G. boninense* pathogenesis, visualization and characterization of host penetration remain elusive.

The genetic transformation of a pathogen provides a means to resolve the many mechanisms underlying disease development (Pardo et al. 2002; Wang et al. 2010). Several successful

transformation protocols have been established for *Ganoderma* spp. (Sharma and Kuhad 2010), including *G. lucidum* (Sun et al. 2001), *G. weberianum* (Zhou et al. 2015), and *G. multipileum* (Chou and Tzean 2016). Various techniques such as electroporation, *Agrobacterium*-mediated transformation (AMT), and biolistic transformation are employed to transform species within the fungal kingdom (Michielse et al. 2005). Despite the robust availability of resources, AMT is highly favored due to cost effectiveness, conserved integration of the target gene (Zhu et al. 2000), and wide choices of explants amenable to the technique. *Agrobacterium tumefaciens*, a gram-negative, pathogenic bacterium, naturally integrates part of its DNA sequence into a host's genome. The transfer DNA (T-DNA) machinery recognizes host phenolics which, in turn, activates its virulence genes to complete the mechanism (Michielse et al. 2005). More than 130 different fungal species, including zygomycetes, ascomycetes, and basidiomycetes, have been amenable to an AMT system, as indicated through successful regeneration of transformants (Ali and Bakkeren 2011; Mora-Lugo et al. 2014). Presently, AMT is used as an integrated tool in modern fungal research even though the protocols are complex and species specific (Wang et al. 2008). Although AMT protocols are applicable for the family Ganodermataceae, none are described for *G. boninense*. The main objective of this study was to develop an efficient AMT system for the pathogenic fungus *G. boninense* to express the green fluorescent protein (GFP) and determine the penetrating structure employed by the pathogen during the early stage of BSR.

MATERIALS AND METHODS

Bacterial strains. *Escherichia coli* strain JM109 was used for large-scale plasmid amplification. Plasmid DNA was extracted using a High-Speed Plasmid Mini Kit (Geneaid Biotech Ltd.) following the manufacturer's instructions. The following *A. tumefaciens* strains retrieved from stock collections (Laboratory of Crop Plantations, Institute of Tropical Agriculture, Universiti Putra Malaysia) were used in cocultivation procedures: LBA4404,

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*The e-Xtra logo stands for "electronic extra" and indicates that two supplementary figures are published online.

EHA101, EHA105, and GV3101. The GV3101 strain was a kind gift from Dr. Hann-Ling Wong, University Tunku Abdul Rahman, Perak, Malaysia. The minimum inhibitory concentration against kanamycin for all transformed strains was 50 µg/ml, except for EHA101, which was 200 µg/ml.

Fungal explants. A virulent strain of *G. boninense* (T10), provided by Applied Agricultural Resources, Selangor, Malaysia, was used for transformation. Three types of explants were utilized in this study: protoplast, basidiospore, and mycelium. Mycelia grown on a nitrocellulose membrane overlaid on malt extract agar (MEA) (Merck) supplemented with 2% lignin alkali (Sigma-Aldrich) was harvested as cubes of 0.5 by 0.5 cm from the leading edge of the colony at day 5 after inoculation. For production of basidiospores, a basidiocarp (mature fruiting body) was artificially obtained in glasshouse conditions (Govender et al. 2016). A fully mature basidiocarp, manifesting a stipe, bracketed cap with visible hymenium on its underside, and deep red coloration on its outer surface, was utilized for subsequent basidiospore isolation. The visible hymenium region of the basidiocarp was cut into small pieces and dropped into deionized water. The mixture was subjected to agitation at 150 rpm for 20 min followed by filtration using glasswool. The resultant filtrate was centrifuged at $1,000 \times g$ for 1 min. The supernatant was directly diluted to the required concentration for the transformation procedure. For protoplast isolation, enzymatic digestion was performed using a commercial lysing enzyme (L1412) from *Trichoderma harzianum* (Sigma-Aldrich). Protoplasts were obtained from 3-day-old mycelia digested in lysing enzyme (10 mg/ml) prepared in 0.6 M potassium chloride and 20 mM dipotassium phosphate buffer (pH 5.4). The mixture was incubated for 3 h at 30°C, then filtered with Whatman paper number 4 (Sigma-Aldrich). The basidiospore and protoplast regeneration assessments were performed prior to the transformation procedure (Govender et al. 2016). Both basidiospore and protoplast suspensions were adjusted to the required concentrations using the hemocytometer.

Plasmid construction. Vector pCAMBIA 1304, provided by Dr. Sreeramanan Rahman (Universiti Sains Malaysia, Pulau Pinang, Malaysia) was modified as described by Sharma and Kuhad, (2010) prior to transformation into *Agrobacterium* strains using the heat-shock method (Wang et al. 2010). Vector p416 ATCC87360 (Addgene) harboring the glyceraldehyde 3-phosphate dehydrogenase (*gpd*) fungal promoter (Mumberg et al. 1995) was excised using a *SacI* and *XbaI* double-enzyme digestion following the manufacturer's instructions (Fast Digest; Thermo Scientific). The binary vector pCAMBIA 1304 was digested with the same enzymes. The double-digested products of p416 were subjected to gel electrophoresis, followed by excision of the 281-bp DNA fragment harboring the *gpd* promoter using the GeneJet Gel Extraction Kit (Thermo Scientific), as described by the manufacturer's protocol. The generated fragment was ligated into the multiple cloning site between the left and right T-DNA borders of pCAMBIA 1304 using the Rapid DNA ligation kit (Thermo Scientific). The resultant modified pCAMBIA 1304 (12,643 bp) was cloned into *E. coli* (JM109) for large-scale amplification and verified via DNA sequencing (First Base).

Growth media and culture conditions. The *Agrobacterium* strains were grown on Luria-Bertani medium amended with kanamycin, as required (50 or 200 µg/ml). Minimal medium (MM) was composed of 10 mM K_2HPO_4 , 10 mM NaH_2PO_4 , 2.5 mM KCl, 2 mM $MgSO_4$, 0.7 mM $CaCl_2$, 9 µM $FeSO_4$, 4 mM $(NH_4)_2SO_4$, and 10 mM glucose, pH 7.0. The induction medium (IM) was prepared by adding the following to MM: 0.5% (wt/vol) glycerol, 250 µM acetosyringone, and 40 mM 2-(N-morpholino) ethanesulfonic acid, pH 5.3. Induction agar was prepared as induction medium with 2% agar. The selection media used depended on the explant source; potato dextrose agar (PDA) for basidiospore, potato glucose agar (PGA) for protoplast, and MEA for mycelium. Selection media were supplemented with cefotaxime at 100 µg/ml and hygromycin

(hyg) B at 200 µg/ml. All induction and selection media for protoplast explants only were further supplemented with 0.6 M sucrose and 20 mM phosphate buffer, which served as the osmotic stabilizer. All chemicals and medium, unless stated otherwise were purchased from Sigma-Aldrich.

Transformation procedure. The *A. tumefaciens* cultures (LBA4404, EHA101, EHA103, and GV3103) were grown in MM supplemented with kanamycin (50 or 200 µg/ml) at 28°C with shaking (150 rpm) overnight. Each culture was centrifuged at $1,000 \times g$ for 5 min; the resultant pellet was suspended in IM supplemented with kanamycin and grown for 8 h under similar conditions. Explants of *G. boninense* were freshly harvested and prepared in deionized water at the following concentrations: mycelia cubes on nitrocellulose membrane (10 cubes/ml) and basidiospores and protoplasts at 10^4 to 10^5 per milliliters each. The *Agrobacterium* (optical density at 600 nm = 0.05 to 0.1) and explant mixture prepared at a 1:100 ratio with all possible combinations was spread on a nitrocellulose membrane overlaid on the induction agar. The plate was incubated at 22°C for 2 days; then, the nitrocellulose membrane was transferred onto a selection agar and grown at 28°C until visible transformant colonies appeared (approximately a week). Transformation efficiency was determined by dividing the number of transformed single colonies on the selection plate by the number of wild-type colonies on the control plate. Nontransformed mycelia, basidiospores, and protoplasts (wild type) were inoculated on nonsupplemented PDA as controls. All transformants were species verified using the *Ganoderma* selective medium, Malaysian Palm Oil Board, Selangor, Malaysia (Ariffin et al. 2000) which forms a brown ring around colonies of *G. boninense* only (Supplementary Fig. S1). In addition, we performed polymerase chain reaction (PCR) for amplification of the *G. boninense* internal transcribed spacer region using the following primers: forward, 5'-gtgggttatagatcgtgtgga-3' and reverse, 5'-ttctaaccgtcccggtattgg-3'.

Because the transformant derived from protoplast explant cocultivated with *Agrobacterium* LBA4404 showed the best transformation efficiency coupled with good GFP fluorescence, we further optimized the following parameters for this particular combination: *Agrobacterium* strain/explant ratio, cocultivation temperature, and cocultivation period. The protoplast suspension was mixed with *Agrobacterium* LBA4404 at 1:1, 1:10, 1:100, and 1:1,000 (*Agrobacterium*/explant) volume ratios. Cocultivation temperature was set at 20, 22, and 26°C for 2 days using the *Agrobacterium* LBA4404 and protoplast explant (1:100) combination. Cocultivation period for *Agrobacterium* LBA4404 and protoplast explant at 1:100 ratio was also evaluated by incubating mixtures at 22°C for 1, 2, 3, 4, and 5 days. All cocultivation mixtures were transferred to nitrocellulose membrane overlaid on induction agar and incubated with the stated modifications using the same transformation procedure. The transformation efficiency was determined as described earlier.

Molecular analysis of *G. boninense* transformants. The integration of T-DNA and gene copy number of GFP was determined for protoplast-LBA4404, basidiospore-GV3101 and mycelia-EHA101-derived transformants via PCR and quantitative (q)PCR. The *G. boninense* transformants and wild-type isolate were grown on nitrocellulose membrane overlaid on selection media and PDA, respectively, for 5 days. The harvested mycelia were ground into fine powders in liquid nitrogen. DNA was extracted using a GeneJet Genomic DNA Purification Kit (Thermo Scientific), following the manufacturer's instructions. In total, 50 ng of fungal genomic DNA and 10 ng of plasmid pCAMBIA1304 were used as template and positive control, respectively, throughout all amplifications. To verify the T-DNA integration, PCR analyses were performed using Hyg^r (Hyg F and Hyg R) and Gus-gfp^r (Gus-gfp F and Gus-gfp R) cassette primers (Table 1). The PCR program was set at 94°C for 5 min; 35 cycles of 94°C for 60 s, 58°C for 30 s, and 72°C for 30 s; and final extension at 72°C for 3 min. PCR Master Mix (2×) (Dream Taq; Thermo Scientific), was fixed at a final

volume of 50 µl with 400 nM each forward and reverse primer. All PCR assays were carried out using thermal cycler C1000 Touch (Bio-Rad), and the PCR products were analyzed on 1.8% agarose gel stained with RedSafe dye (Intron Biotechnology).

As for the determination of gene copy number, genomic DNA extracted from both *G. boninense* wild-type isolate and transformants were used as templates, while plasmid pCambia1304 was used as the positive control. The PCR product of the GFP fragment was run parallel for extrapolation of standard curve. The GFP fragment number in each transformant was determined as described by Dorak (2007). The number of GFP molecular copies for the standard curve was determined by dividing the concentration of DNA product (nanograms) by mass. A standard curve constructed from serial dilutions (10-fold differences) was used to determine GFP copy number in *G. boninense* transformants. The qPCR was performed in triplicate with 80 ng of fungal genomic DNA in a 20-µl reaction using a Green Real-Time PCR Master Mix (2×) kit (RealMOD) and CFX96 Touch Real-Time PCR Detection System (Bio-Rad). The final concentration of primers was 500 nM, and the reaction yielded a 179-bp DNA fragment. All reactions were performed under the following conditions: 94°C for 5 min; 35 cycles of 94°C for 60 s, 60°C for 30 s, and 72°C for 30 s; and final extension at 72°C for 3 min. Melt-curve analysis was performed to verify the absence of primer-dimer products.

β-Glucuronidase-GFP expression in *G. boninense* transformants. Fluorescence associated with GFP in *G. boninense* transformants was monitored using an Olympus FV1000 confocal laser-scanning microscope (Olympus America Inc.). Thin mycelia sections (6 by 6 mm) from the leading edge of 5-day-old transformant growing on selective media were mounted in water prior to microscopic examination.

Artificial infection of oil palm plantlets. The protoplast LBA4404-derived *G. boninense* transformant was grown on PGA for 5 days. Oil palm roots harvested from 5-year-old trees were utilized as substrate for preparation of inoculum. Oil palm roots (2 to 3 cm in diameter) were washed thoroughly, dried at 80°C for 3 days, and ground into a fine powder. Solidifying media composed of glucose (35 g/liter) and 5% Bacto agar were added to each 100 g of oil palm root powder at a 1:1 (vol/wt) ratio. The mixture was autoclaved at 121°C for 20 min and left to cool at room temperature. The semisolid media was shaped into balls of 2 to 3 cm in size using a sterilized metal spatula. Approximately two to three pieces of mycelia cubes (0.5 by 0.5 mm) were added to each ball. The balls were incubated in the dark at 26 ± 2°C for a week. Only balls fully colonized with mycelia were utilized for subsequent artificial infection of oil palm plantlet. Oil palm plantlets (5 weeks old) were grown under sterile tissue culture conditions in Gamborg B medium supplemented with 1-naphthaleneacetic acid at 0.1 mg/liter (Tarmizi 2002). Artificial inoculation of oil palm roots was performed on plantlets with a visible mass of roots. The edge of the oil palm plantlet root system was partially lifted out from the growing medium to create aeration. Balls of inocula were attached on aerated root surface by exerting a gentle pressure on the site of infection to secure the position of the inoculum on the roots. All infected plantlets were grown at 27°C in the dark, with shaking at 90 rpm. At 1 and 2 weeks postinoculation, root cross-sections

(2 to 5 mm) and root surface longitudinal sections of the oil palm plantlets were prepared for microscopic examination using the confocal laser microscopy equipped with filter blocks for GFP (488 nm) and root autofluorescence (546 nm), as described by Li et al. (2013). The broadband fiber connection was set at 415- to 635-nm lasers with a single fiber-type combiner. All digital images obtained were processed by OlyVIA, version 3.

RESULTS

AMT of *G. boninense*. We tested three explant types (protoplast, basidiospore, and mycelia) and four *Agrobacterium* strains (LBA4404, EHA101, EHA105, and GV3103) at all possible combinations under the following conditions: cocultivation period of 2 days at 22°C, with *Agrobacterium*/explant ratio set at 1:100. Depending on the explant types and *Agrobacterium* strains, transformation efficiency was 2 to 62% (Table 2). Of the three explant types utilized in this study, and using the conditions described above, protoplasts cocultivated with *Agrobacterium* strain LBA4404 showed the highest transformation efficiency (62%), followed by basidiospore cocultivated with *Agrobacterium* strain GV3103 (52%). In comparison with protoplast and basidiospore explant types, mycelium was least efficient; cocultivation with *Agrobacterium* EHA101, relative to other strains, showed the highest transformation efficiency (12%).

Cocultivation of protoplasts with the four different *Agrobacterium* strains yielded transformation efficiency of 35 to 62%, which was relatively higher than basidiospore and mycelium at 40 to 52 and 2 to 12%, respectively (Table 2). The effect of *Agrobacterium* strain on transformation efficiency differed greatly with the different explants types used for cocultivation. Regeneration of transformants from basidiospores and protoplasts required approximately 2 weeks for formation of visible fungal colonies, and five subsequent subcultures regenerated successfully. In contrast, though cocultivation of *Agrobacterium* and mycelia showed successful transformation, poor regeneration of subcultures from the primary transformant was evident (data not shown).

Based on the results obtained from Table 2, we selected the most efficient explant-*Agrobacterium* pairing (evident with the highest transformation efficiency), protoplast and *Agrobacterium* LBA4404, for further optimization of the following parameters: cocultivation period, cocultivation temperature, and *Agrobacterium*/explant ratios. Transformation efficiency was relatively good (62%) at the 1:100 *Agrobacterium*/explant ratio. As the concentration of *Agrobacterium* increased by 10-fold increments, transformation efficiency decreased from 12% at 1:10 to 0% at 1:1

TABLE 1. Primer sequences for polymerase chain reaction (PCR) and quantitative PCR analysis of wild and transformant *Ganoderma boninense*

Number	Primer name	Sequence (5'–3')	Product size (bp)
1	Hyg F	GCTCTGATAGAGTTGGTC	806
	Hyg R	CGTCTGTCGAGAAGTTT	...
2	Gus-gfp F	GTCAAGTTTGAGGGAGAC	1,395
	Gus-gfp R	CATCTCTTCAGCGTAAGG	...
3	Gfp F	TTTCTGTCAGTGGAGAGG	179
	Gfp R	GCTCTGAAGAAGTCGTG	...

TABLE 2. Transformation efficiency (TE) of *Ganoderma boninense* at all possible combinations of *Agrobacterium* strains and fungal explants^a

<i>G. boninense</i> explant	Strain	TE (%) ^b	Regen ^c
Protoplast	LBA4404	62	Yes
	GV3101	46	Yes
	EHA101	32	Yes
	EHA105	35	Yes
	LBA4404	40	Yes
Basidiospore	GV3101	52	Yes
	EHA101	44	Yes
	EHA105	48	Yes
	LBA4404	2	No
	GV3101	5	No
Mycelium	EHA101	12	No
	EHA105	7	No

^a All transformations were performed using the 1:100 *Agrobacterium*/explant mixture cocultivated on a nitrocellulose membrane overlaid on an induction medium for 2 days at 22°C, then transferred onto a selection medium and incubated at 28°C for a week.

^b Verified by hygromycin B selection.

^c Regeneration at subsequent subcultures.

Agrobacterium/explant ratio (Fig. 1A). At 1:1,000 *Agrobacterium*/explant ratio, transformation efficiency was zero. A cocultivation at 22°C for 2 days showed the highest transformation efficiency at 63%, followed by 3 days with a transformation efficiency of 31% (Fig. 1B). A period of 2 to 3 days was ideal for the transfer of T-DNA without compromising its ability to regenerate latter. A short period of 1 day showed poor (7%) transformation efficiency, while no transformants were obtained for incubation periods of 4 to 5 days (data not shown). Cocultivation temperatures of 22, 24, and 26°C were evaluated. At 22 and 24°C, the transformation efficiency was 62 and 60%, respectively. However, at 26°C, transformation was not successful (Fig. 1C).

Selection media affected the regeneration of transformants. Wild-type *G. boninense* basidiospores, protoplasts, and mycelia could be grown on PDA. However, the protoplast-derived

transformants were able to produce mycelia better when grown on PGA compared with PDA (Supplementary Fig. S2). The regeneration efficiency for mycelium-derived transformants could not be further optimized.

Transformant: Integration and expression analysis. The binary vector pCambia1304 allows dual selection of transformants based on β -glucuronidase (GUS) staining and GFP fluorescence. In this study, only the GFP activity was monitored.

Successful integration of marker genes (*Hyg* and *gus-gfp* fusion) in transformants derived from protoplast-*Agrobacterium* LBA4404, basidiospore-GV3101, and mycelium-EHA101 was detected in the F1 generation (Fig. 2).

The GFP expression by transformants derived from all three explant types was generally inconsistent (Fig. 3). Although GFP distribution was not documented quantitatively, based on our observations, we believe the GFP expression was more uniform in protoplast-*Agrobacterium* LBA4404-derived transformants (Fig. 3A) compared with basidiospore-*Agrobacterium* GV3101- (Fig. 3B) and mycelium-*Agrobacterium* EHA101-derived transformants (Fig. 3C). The GFP expressions of the protoplast- and basidiospore-derived transformants remained stable for five subsequent subcultures. All transformants subjected to confocal microscopy were harvested as cubes (0.5 by 0.5 cm) from the leading edge of its axenic culture.

Copy number of the GFP fragment from the protoplast-*Agrobacterium* LBA4404-derived transformant was the lowest, with an average of 10, followed by basidiospore-*Agrobacterium* GV3101, with an average of 22 and mycelium-*Agrobacterium* EHA101-derived transformant, with an average of 32 (Fig. 4). Monitoring of early-stage infection of oil palm seedlings was performed using transformants derived from the protoplast-*Agrobacterium* LBA4404 combination only.

GFP marker in *G. boninense* transformant to discern the early stage infection of BSR. The protoplast-*Agrobacterium* LBA4404-derived transformant was selected to monitor the early stage of oil palm seedling infection. When inoculated on balls of oil palm root powder, the transformant showed complete colonization

Fig. 1. Cocultivation conditions for *Ganoderma boninense* transformant derived from *Agrobacterium* LBA4404 and protoplast mixture. Transformation efficiency at different **A**, cocultivation periods; **B**, cocultivation temperatures; and **C**, explant/*Agrobacterium* ratios. All transformation conditions used were as follows, unless stated otherwise: *Agrobacterium* LBA4404/protoplast (1:100) mixture incubated for 2 days at 22°C on induction medium prior to selection.

Fig. 2. Polymerase chain reaction detection of the *gus-gfp* fusion gene within *Ganoderma boninense* transformants derived from different explant type-*Agrobacterium* strain combinations. Lane M, DNA size marker (1.5 kb); lane 1, positive control plasmid pCambia 1304; lane 2, negative control wild-type *G. boninense*; lane 3, basidiospore-*Agrobacterium* GV3103-derived transformant; lane 4, protoplast-*Agrobacterium* LBA4404-derived transformant; lane 5, mycelia-*Agrobacterium* EHA101-derived transformant. Transformation was conducted with a 1:100 *Agrobacterium*/explant mixture cocultivated on a nitrocellulose membrane overlaid on an induction medium for 2 days at 22°C, then transferred onto a selection medium and incubated at 28°C for a week.

by day 6 after inoculation (Fig. 5A). The ball-like inoculum appeared to be firmly attached to the root surface of the oil palm plantlets (Fig. 5B) throughout the experiment. At week 1 post-inoculation, the oil palm root surface showed no visible symptoms. At week 2 postinoculation, visible fungal hyphae were observed on the root surface. In root cross-sections, the transformant expressing the GFP could be observed. Root autofluorescence showed yellow coloration at the exodermis layer (Fig. 5C). Longitudinal root surface sections and root cross-sections were prepared from and within the area (<2 cm) of fungal attachments (Fig. 5D).

G. boninense microhyphae, detected as green fluorescence, were observed penetrating the root exodermis layer at random positions (Fig. 5E). The pathogen was found entering in a longitudinal fashion from the epidermis into the exodermis and cortical tissues of the root tissue (Fig. 5F). It appears that penetration by *G. boninense* begins with the morphogenesis of a needle-like microhyphae. Upon

evaluation of the longitudinal root-surface sections (collected at the point of inoculation) under confocal microscopy, the needle-like microhyphae could not be resolved because the GFP fluorescence was too high due to the presence of a dense network of mycelia. However, within 10 to 15 mm from the point of fungal attachment, the longitudinal root-surface sections showed the presence of needle-like microhyphae in the absence of visible fungal attachment.

DISCUSSION

We developed an AMT protocol for *G. boninense* based on a revised cocultivation procedure described earlier (Sun et al. 2001). Although this protocol was not compatible for transforming mycelia, both protoplasts and basidiospores could be transformed efficiently. However, it took less time to obtain protoplasts from

Fig. 3. Green fluorescent protein-expressing *Ganoderma boninense* from different explant type-*Agrobacterium* strain combinations as observed with confocal laser microscopy (left) and bright-field microscopy (right) (scale bar = 100 μ m). Images captured for the following: **A**, protoplast-*Agrobacterium* LBA4404-derived hyphae; **B**, basidiospore-*Agrobacterium* GV3101-derived hyphae; and **C**, mycelia-*Agrobacterium* EHA101-derived hyphae.

mycelia cultures than to obtain basidiospores from an artificially produced basidiocarp (approximately 5 to 10 weeks). The T-DNA transfer, which was only possible in the presence of the *gpd* promoter of fungal origin, was comparable with several other

studies (Kuo et al. 2004; Sharma and Kuhad 2010). The *Agrobacterium*/explant ratio was pivotal to transformation efficiency. In any treatment with a high ratio of either *Agrobacterium* (1:1) or explant (1:1,000), transformants failed to regenerate into mycelia. During cocultivation, which excluded the amendment of cefotaxime, an increased *Agrobacterium* population may have suffocated explants, resulting in poor transformation efficiency. Likewise, an increased fungal population may have interfered with the T-DNA integration into fungal nuclear genome. Our results were comparable with those of Shi et al. (2012), who demonstrated use of a low *Agrobacterium*/explant ratio for increased transformation efficiency of *G. lucidum*.

Visualization of fungal structures within roots has been demonstrated using fluorescent pathogens harboring selective markers such as GFP and GUS (Rajasekaran et al. 2008; Vallad and Subbarao 2008; Zhang et al. 2013) and our study demonstrated the same with *G. boninense*. The BSR time course infection has been well characterized based on cellular changes on oil palm root tissues (Rees et al. 2009). However, in-depth penetration was not evident. Because the disease is asymptomatic during the initial stage of colonization, resolving the penetration event using popular techniques such as conventional staining and transmission electron microscopy did not seem to be a feasible approach. The initial population size of the pathogen during host infection would be relatively small and probably insufficient for efficient staining.

Fig. 4. Copy number of green fluorescent protein (GFP) transcripts in *Ganoderma boninense* transformants from different explant type-*Agrobacterium* strain combinations: protoplast-LBA4404, basidiospore-GV3103, and mycelia-EHA101. The bars represent standard deviation computed from four biological replicates per treatment. All transformations were performed using *Agrobacterium*/explant at a 1:100 ratio, cocultivated on an induction medium for 2 days at 22°C prior to selection.

Fig. 5. Green fluorescent protein-expressing *Ganoderma boninense* for artificial infection of oil palm plantlets. **A**, Ball-like root medium (left) and *G. boninense* transformant (derived from protoplast-*Agrobacterium* LBA4404 combination) colonized inoculum (right). **B**, Noninoculated (control) oil palm plantlets (left) and infected oil palm plantlets (right) at week 1 postinoculation. Red arrow and circle indicate the attached inoculum. **C**, Root cross section (control) indicates autofluorescence (yellow signal) of the exodermis (scale bar = 250 μ m). **D**, Infected tissue for longitudinal root surface (circled area) and cross-sectioning (at fungal attachment). **E**, Longitudinal penetration by microhyphae from epidermis into exodermis and cortex (scale bar = 180 μ m). Red arrows indicate the needle-like microhyphae (green fluorescences). **F**, Microhyphae (phase contrast) invasion at the cortex region of the *G. boninense*-infected root tissues (scale bar = 100 μ m).

Moreover, because the pathogen is found within the root tissues, selective tissue-specific dyes are required to aid in localization of the hyaline hyphae. The described transformation protocol for *G. boninense* enabled the production of GFP-expressing pathogens with good fluorescence and allowed microscopic visualization of small amounts of fungal tissue.

The exodermis layer of the oil palm root was punctured at random positions by GFP-expressing *G. boninense* needle-like microhyphae (Fig. 5E). The exodermis layer was observed to autofluoresce due to the presence of lignin and other phenolic compounds. Although these compounds have been documented as key components in the oil palm defense mechanisms (Paterson 2007), the pathogen (microhyphae) appeared to breach the host's first line of defense effortlessly. Morphogenesis of microhyphae in the fungal kingdom has served various physiological roles. In *Neurospora crassa* (Trevithick and Galsworthy 1977) and *Fusarium oxysporum* (Charest et al. 2004), microhyphae were found triggered during growth in the presence of inhibitors. In contrast, *Phellinus noxius* (Nicole et al. 1995) utilized microhyphae for invasive cell wall colonization during wood degradation. Our study demonstrated apparent morphogenesis of microhyphae during the early-stage infection of BSR. The particularities of the microhyphae utilized by *G. boninense*—thin fungal cells with discontinuous walls at extended long distances in the host—were in agreement with other colonization studies (Charest et al. 2004; Ouellette and Baayen 2000; Ouellette et al. 1999, 2002). Microhyphae with thin, imperceptible walls with only certain normal cytoplasmic components (Ouellette et al. 1999) are model organisms for enzyme secretion studies (Ouellette and Baayen 2000). *G. boninense* is a white rot fungi which uses lignin-modifying enzymes such as laccase, manganese peroxidase, and lignin peroxidase for decomposition of the wood components (Lundell et al. 2010). Although the BSR infection begins with a tight host–pathogen contact, the enzymes (large globular proteins produced by the pathogen at the site of attachment) fail to diffuse through the plant cell wall. The *G. boninense* microhyphae, which efficiently punctures the host cell wall, bypasses the need for enzyme diffusion.

Breeding for resistance is a far-reaching approach for managing BSR disease. Host resistance corresponds to either an arrested fungal penetration into the host cell or the complete inhibition of fungal colonization at the expense of the host (Hammond-Kosack and Jones 1997). The former impedes host–pathogen surface contact whereas the latter requires a systemic host defense response, functional only in the presence of a resistant genetic make-up. Impeding the penetration event would serve as a better BSR knock-out strategy, as opposed to the subsequent colonization event. For instance, application of compounds which are antagonist against the penetration structure could ultimately block the pathogen–host contact. Resistance to BSR is yet to be reported. However, planting materials with variable degrees of tolerance have been characterized: ‘Dura’ × Dura and ‘Deli’ × Deli are most susceptible to BSR, whereas Dura × ‘Psifera’ and ‘Zaire’ × ‘Cameroon’ are considerably tolerant to BSR (Idris and Ariffin 2004). Genetically modified oil palm promises resistance; though it is possible, nevertheless, it is rather impractical to perform large-scale replanting of already existing plantations. Thus, resolving the penetration event contributes to efficient management strategies which could ultimately result in significant economic impacts to the oil palm industry. Our study is the first to demonstrate a reliable and highly efficient system for transformation of *G. boninense*. The results obtained demonstrated the applicability of GFP-expressing *G. boninense* as a model system for the study of BSR. The GFP-expressing transformant, together with the artificial infection of oil palm plantlets, could also be used for real-time assessment of fungicides, bio-control agents, and other strategies to suppress BSR disease. Preliminary results could be achieved in a shorter period of time in comparison with conventional glasshouse and field trials, which are time consuming.

ACKNOWLEDGMENTS

We thank the Ministry of Higher Education (MOHE), Malaysia for providing Fundamental Research Grant Scheme, and Applied Agricultural Resources, Petaling Jaya, Selangor for partially financing this study. We thank I. A. Seman from the Malaysian Palm Oil Board (MPOB) for providing oil palm plantlets and M. Mahmood (Universiti Putra Malaysia) and W. H. Ling (Universiti Tunku Abdul Rahman, Malaysia) for their technical support. N. Govender was supported by a MyBrain Ph.D. scholarship sponsored by MOHE, Malaysia.

LITERATURE CITED

- Ali, S., and Bakkeren, S. 2011. Introduction of large DNA inserts into the barley pathogenic fungus, *Ustilago hordei*, via recombined binary BAC vectors and *Agrobacterium*-mediated transformation. *Curr. Genet.* 57:63-73.
- Ariffin, D., Idris, A. S., and Singh, G. 2000. Status of Ganoderma in oil palm. Pages 49-68 in: *Ganoderma Diseases of Perennial Crops*. J. Flood, P. D. Bridge, and M. Holderness, eds. CABI Publishing, Wallingford, UK.
- Charest, P. M., Ouellette, G. B., Blais, P., and Chamberl, H. 2004. Irregular growth forms and cell wall modifications, polygalacturonase detection, and endocell formation in *Fusarium oxysporum* f. sp. *radicis-lycopersici* infecting tomato plants, as studied ultrastructurally and cytochemically. *Mycol. Prog.* 3:137-150.
- Chou, T. H., and Tzean, S. S. 2016. Protoplasting, regeneration and transformation of medicinal mushroom *Ganoderma multipileum* using succinate dehydrogenase mutation gene as a selection marker. *Ann. Microbiol.* 66:111-120.
- Dorak, M. T. 2007. Real-time PCR. Online publication. Taylor and Francis Group. <http://www.gene-quantification.com/dorak-book-real-time-pcr-2006.pdf>
- Govender, N. T., Mahmood, M., Seman, I. A., and Wong, M. Y. 2016. Basidiospore and protoplast regeneration from raised fruiting bodies of pathogenic *Ganoderma boninense*. *Pol. J. Microbiol.* 65:383-388.
- Hammond-Kosack, K. E., and Jones, J. D. G. 1997. Plant disease resistance genes. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48:575-607.
- Ho, Y. W., and Nawawi, A. 1985. *Ganoderma boninense* Pat. from basal stem rot of oil palm (*Elaeis guineensis*) in Peninsular Malaysia. *Pertanika* 8:425-428.
- Idris, A. S., and Ariffin, D. 2004. Basal Stem Rot-Biology, Detection and Control. Paper number 9 in: *Int. Conf. Pest Dis. Importance Palm Industry*, Kuala Lumpur, Malaysia.
- Kuo, C. Y., Chou, S. Y., and Huang, C. T. 2004. Cloning of glyceraldehydes-3-phosphate dehydrogenase gene and use of the gpd promoter for transformation in *Flammulina velutipes*. *Appl. Microbiol. Biotechnol.* 65:593-599.
- Li, C. Q., Shao, J. F., Wang, Y. J., Li, W. B., Guo, D. J., Yan, B., Xia, Y., and Peng, M. 2013. Analysis of banana transcriptome and global gene expression profiles in banana roots in response to infection by race 1 and tropical race 4 of *Fusarium oxysporum* f. sp. *cubense*. *BMC Genomics* 14:851.
- Lundell, T. K., Makela, M. R., and Hilden, K. 2010. Lignin-modifying enzymes in filamentous basidiomycetes-ecological, functional and phylogenetic review. *J. Basic Microbiol.* 50:5-20.
- Michielse, C. B., Hooykaas, P. J. J., van den Hondel, C. A. M. J. J., and Ram, A. F. J. 2005. *Agrobacterium*-mediated transformation as a tool for functional genomics in fungi. *Curr. Genet.* 48:1-17.
- Mora-Lugo, R., Zimmermann, J., Rizk, A. M., and Fernandez-Lahore, M. 2014. Development of a transformation system *Aspergillus sojae* based on the *Agrobacterium tumefaciens*-mediated approach. *BMC Microbiol.* 14:247.
- Mumberg, D., Muller, R., and Funk, M. 1995. Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene* 156:119-122.
- Nicole, M., Chamberland, H., Rioux, D., Xixuan, X., Blanchette, R. A., Geiger, J. P., and Ouellette, G. B. 1995. Wood degradation by *Phellinus noxius*: Ultrastructure and cytochemistry. *Can. J. Microbiol.* 41:253-265.
- Ouellette, G. B., and Baayen, R. P. 2000. Peculiar structures occurring in vessel walls of the susceptible carnation cultivar Early Sam infected with *Fusarium oxysporum* f. sp. *dianthi*. *Can. J. Bot.* 78:270-277.
- Ouellette, G. B., Baayen, R. P., Simard, M., and Rioux, D. 1999. Ultrastructural and cytochemical study of colonization of xylem vessel elements of susceptible and resistant *Dianthus caryophyllus* by *Fusarium oxysporum* f. sp. *dianthi*. *Can. J. Bot.* 77:644-663.
- Ouellette, G. B., Baayen, R. P., Simard, M., and Rioux, D. 2002. Reactions of paratracheal cells of resistant and susceptible carnation (*Dianthus caryophyllus*) cultivars to vascular invasion by *Fusarium oxysporum* f. sp. *dianthi*. *New Phytol.* 156:113-128.
- Pardo, A. G., Hanif, M., Raudaskoski, M., and Gorfer, M. 2002. Genetic transformation of ectomycorrhizal fungi mediated by *Agrobacterium tumefaciens*. *Mycol. Res.* 106:132-137.

- Paterson, R. R. M. 2007. Ganoderma disease of oil palm—A white rot perspective necessary for integrated control. *Crop Prot.* 26:1369-1376.
- Rajasekaran, K., Cary, J. W., Cotty, P. J., and Cleveland, T. E. 2008. Development of a GFP-expressing *Aspergillus flavus* strain to study fungal invasion, colonization, and resistance in cottonseed. *Mycopathologia* 165:89-97.
- Rees, R. W., Flood, J., Hassan, Y., Potterd, U., and Cooper, R. M. 2009. Basal stem rot of oil palm (*Elaeis guineensis*): Mode of infection and lower stem invasion by *Ganoderma boninense*. *Plant Pathol.* 58:982-989.
- Sharma, K. K., and Kuhad, R. C. 2010. Genetic transformation of lignin degrading fungi facilitated by *Agrobacterium tumefaciens*. *BMC Biotechnol.* 10:67.
- Shi, L., Fang, X., Li, M. J., Mu, D., Ren, A., Tan, Q., and Zhao, M. 2012. Development of a simple and efficient transformation system for the basidiomycetous medicinal fungus *Ganoderma lucidum*. *World J. Microbiol. Biotechnol.* 28:283-291.
- Sun, L., Cai, H., Xu, W. H., Hu, Y., Gao, Y., and Lin, Z. 2001. Efficient transformation of the medicinal mushroom *Ganoderma lucidum*. *Plant Mol. Biol. Rep.* 19:383-384.
- Sundram, S., Sariah, M., Idris, A. S., and Radziah, O. 2011. Symbiotic interaction of endophytic bacteria with arbuscular mycorrhizal fungi and its antagonistic effect on *Ganoderma boninense*. *J. Microbiol.* 49:551-557.
- Tarmizi, A. H. 2002. Oil palm liquid culture—MPOB Protocol. MPOB Information Series. ISSN 1511-7871. Malaysian Palm Oil Board, Bangi Selangor, Malaysia.
- Thompson, A. 1931. Stem rot of the oil palm in Malaya. *Bull. Dep. Agric. Sci.* Ser. 23. Department of Agriculture, Malaysia.
- Trevithick, J. R., and Galsworthy, P. R. 1977. Morphology of slime variants of *Neurospora crassa* growing on a glass surface in liquid medium. *Arch. Microbiol.* 115:109-118.
- Turner, P. D. 1981. Page 280 in: *Oil Palm Diseases and Disorders*. Oxford University Press, Kuala Lumpur, Malaysia.
- Turner, P. D., and Gillbanks, R. A. 2003. *Oil Palm Cultivation and Management*. The Incorporated Society of Planters, Kuala Lumpur, Malaysia.
- Utomo, C., Werner, S., Niepold, F., and Deising, H. B. 2005. Identification of *Ganoderma*, the causal agent of basal stem rot disease in oil palm using a molecular method. *Mycopathologia* 159:159-170.
- Vallad, G. E., and Subbarao, K. V. 2008. Colonization of resistant and susceptible lettuce cultivars by a green fluorescent protein-tagged isolate of *Verticillium dahliae*. *Phytopathology* 98:871-885.
- Wang, J., Guo, L. Q., Zhang, K., and Lin, J. F. 2008. Highly efficient *Agrobacterium*-mediated transformation of *Volvariella volvacea*. *Bioresour. Technol.* 99:8524-8527.
- Wang, Y., DiGiustini, S., Wang, T. T., Bohlmann, J., and Breuil, C. 2010. *Agrobacterium*-mediated gene disruption using split-marker in *Grosmanina clavigera*, a mountain pine beetle associated pathogen. *Curr. Genet.* 56: 297-307.
- Zhang, W. W., Jiang, T. F., Cui, X., Qi, F. J., and Jian, G. L. 2013. Colonization in cotton plants by a green fluorescent protein labelled strain of *Verticillium dahlia*. *Eur. J. Plant Pathol.* 135:867-876.
- Zhou, Y. P., Chen, M. H., Lu, J. J., Kang, X., Chen, Q. H., Huang, X. L., and Tian, C. E. 2015. A simple and efficient genetic transformation method of *Ganoderma weberianum*. *Folia Microbiol. (Praha)* 60:417-423.
- Zhu, J., Oger, P. M., Schrammeijer, B., Hooykaas, P., Farrand, S. K., and Winnans, S. 2000. The bases of crown gall tumorigenesis. *J. Bacteriol.* 182: 3885-3895.