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

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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 necrotrophic 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 electroproporation, 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 amendable 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 amendable 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,
excised using a ATCC87360 (Addgene) harboring the glyceraldehyde 3-phosphate using the heat-shock method (Wang et al. 2010). Vector p416 were supplemented with cefotaxime at 100 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 x 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 lyzing 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. Seeramanan 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 p146 ATCC87360 (Addgene) harboring the glyceraldehyde 3-phosphate dehydrogenase (gpd) fungal promoter (Mumberg et al. 1995) was excised using a Sacl and Xbal 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 pCAMBIA 1304 with 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 K2HPO4, 10 mM Na2HPO4, 2.5 mM KCl, 2 mM MgSO4, 0.7 mM CaCl2, 9 µM FeSO4, 4 mM (NH4)2SO4, 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 x 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 10^6 to 10^7 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’-gttggttagatgctgtagga-3’ and reverse, 5’-tcaacgctcggattg-3’.

Because the transformant derived from protoplast explant cocultivated with Agrobacterium LBA4404 showed the best transformation efficiency coupled with good GFP florescence, we further optimized the following parameters for this particular combination: Agrobacterium strain/explant ratio, co-cultivation temperature, and co-cultivation period. The protoplast suspension was mixed with Agrobacterium LBA4404 at 1:1, 1:10, 1:100, and 1:1,000 (Agrobacterium/explant) volume ratios. Co-cultivation temperature was set at 20, 22, and 26°C for 2 days using the Agrobacterium LBA4404 and protoplast explant (1:100) combination. Co-cultivation 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 (Hyg F and Hyg R) and Gus-gfp (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 (2x) (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 to 3 cm in diameter) were washed thoroughly, dried at 80°C for 5 days. Oil palm roots harvested from 5-year-old trees were incubated at 28°C in the dark, with shaking at 90 rpm. At 1 and 2 weeks postinoculation, root cross-sections

**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 2. Transformation efficiency (TE) of *Ganoderma boninense* at all possible combinations of *Agrobacterium* strains and fungal explanta

<table>
<thead>
<tr>
<th>G. boninense explant</th>
<th>Strain</th>
<th>TE (%)b</th>
<th>Regenc</th>
</tr>
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<tbody>
<tr>
<td>Protoplast</td>
<td>LBA4404</td>
<td>62</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>GV3101</td>
<td>46</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>EHA101</td>
<td>32</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>EHA105</td>
<td>35</td>
<td>Yes</td>
</tr>
<tr>
<td>Basidiospore</td>
<td>LBA4404</td>
<td>40</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>GV3101</td>
<td>52</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>EHA101</td>
<td>44</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>EHA105</td>
<td>48</td>
<td>Yes</td>
</tr>
<tr>
<td>Mycelium</td>
<td>LBA4404</td>
<td>2</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>GV3101</td>
<td>5</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>EHA101</td>
<td>12</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>EHA105</td>
<td>7</td>
<td>No</td>
</tr>
</tbody>
</table>

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.

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

<table>
<thead>
<tr>
<th>Number</th>
<th>Primer name</th>
<th>Sequence (5′–3′)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hyg F</td>
<td>GCTCTGATAGATTGTC</td>
<td>806</td>
</tr>
<tr>
<td></td>
<td>Hyg R</td>
<td>CGTCTGCGAGAGTTT</td>
<td>...</td>
</tr>
<tr>
<td>2</td>
<td>GUS-gfp F</td>
<td>GTCAGATTGAGAGGAG</td>
<td>1,395</td>
</tr>
<tr>
<td></td>
<td>GUS-gfp R</td>
<td>CATCTCCTCTGAGGAG</td>
<td>...</td>
</tr>
<tr>
<td>3</td>
<td>Gfp F</td>
<td>TTTCTGAGATTGAGGAG</td>
<td>179</td>
</tr>
<tr>
<td></td>
<td>Gfp R</td>
<td>GCTCTGAGAGGAGCTG</td>
<td>...</td>
</tr>
</tbody>
</table>
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*) 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.

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**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.
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 post-inoculation, 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...
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-Agro*-bacterium* 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 µm). 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 µm). 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 µm).
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 Galloway 1977) and Fusarium oxysporum (Charest et al. 2004), microhyphae were found triggered during growth in the presence of inhibitors. In contrast, Phellinus noxius (Nico 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 fungus 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 × ‘Psiferá’ 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.

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LITERATURE CITED


