SHORT COMMUNICATION

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A PARTIAL-LENGTH CYCLOPHILIN-ENCODING (*cyp*) cDNA ISOLATED FROM *Ganoderma boninense*

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ABSTRACT

Oil palm is subjected to various pests and diseases, one of the most important being basal stem rot (BSR) disease which is caused by a few species of Ganoderma. Ganoderma *is a white rot fungus that attacks oil palm around the world. During plant infection, a few genes are highly expressed in the fungus and have been linked to fungal pathogenicity. One of these genes is the cyclophilin-encoding gene (*cyp*). In this study, degenerate primers have been designed based on the conserved regions of the gene. PCR amplification successfully amplified a 212 bp DNA fragment from* Ganoderma boninense *cDNAs. The DNA sequence analysis showed that the fragment has about 80% similarity to other plant pathogenic fungal* cyp *genes. The translated amino acid sequence was also highly identical to other plant pathogenic fungal cyclophilin. Conserve domain search results show that the obtained sequence has similarity to the cyclophilin_ABH-like domain.*

Keywords: *Ganoderma boninense*, fungal pathogenicity, cyclophilin, basal stem rot (BSR) disease, cDNA.

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INTRODUCTION

Oil palm is a major crop that grows in the tropical areas, especially in Southeast Asia. Malaysia and Indonesia are the major palm oil producers and exporters in the world. Palm oil is used worldwide in the production of food, cosmetics, pharmaceuticals and, recently, biodiesel (Kalam and Masjuki, 2002; Corley and Tinker, 2003). Palm oil and its products have been exported and consumed in over 150 countries around the world. Thus, oil palm has played important roles in the Malaysian economy as well as in the country's rural area development (Chin, 2008).

Oil palm is subjected to various devastating diseases such as basal stem rot (BSR), vascular wilt, spear rot-bud rot, sudden wither and red ring (Corley and Tinker, 2003). BSR disease which is caused by *Ganoderma* is the main concern in Southeast Asia. Four species of *Ganoderma* that infect oil palm have been identified. They are *G. boninense, G. zonatum, G. miniatocinctum* and *G. tornatum* (Idris *et al.*, 2000). *G. boninense* is the most aggressive among the identified species, while *G. tornatum* is only found on dead palms, and presumably saprophytic.

Infected palms have rotted basal trunk tissues, wilting leaves and show malnutrition. The disease can also be identified from the dry rot, the presence of the small white button- and bracket-shaped basidiomata of *Ganoderma* at the basal palm trunk (Idris *et al.*, 2009). BSR disease causes a direct reduction in the number of standing palms in the field. Furthermore, the disease also causes reductions in the number and weight of fruit bunches from the standing diseased palms (Turner, 1981). Initially, BSR disease was not economically harmful as it only attacked older palms over 25 years of age (Thompson, 1931). However, during the past 30 years, the disease has started to infect

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younger palms as young as one or two years after planting (Turner, 1981; Singh, 1990). This has resulted in reports on the death of more than 80% of the palms when they were only halfway through their normal life cycle (Idris and Ariffin, 2004). Singh (1991) has shown that 31%-67% of disease incidence in the field may cause around a 26%-46% yield reduction in fresh fruit bunches (FFB).

Fungi cause more plant diseases than any other group of microbes (Lu *et al*., 2003). Research on the interaction of pathogenic fungi and their hosts have been carried out in recent years (Horbach *et al*., 2010). The presence of a common or unique set of virulence determinants has been hypothesised to be essential for fungal pathogenicity (Lu *et al*., 2003). This information is essential for the development of a systematic and effective approach against the disease. However, the knowledge and understanding of the fundamental infection mechanism and biochemical processes of *Ganoderma* are still obscure (Paterson *et al*., 2009).

Cyclophilins (CYP) are peptidyl prolyl *cis-trans* isomerases which are a highly conserved family of proteins in bacteria, fungi, plants and animals. The enzymatic function of the cyclophilins is to facilitate and accelerate protein-folding by catalysing the peptide bond isomerisation that precedes proline residues (Fischer and Schmid, 1990). Cyclophilins have also been implicated in various cellular processes, including environmental stress response, control of the cell cycle, regulation of calcium signaling, and control of transcriptional repression (Sykes *et al*., 1993; Lu *et al*., 1996; Andreeva *et al*., 1999; Gothel and Marahiel, 1999; Arevalo-Rodriguez *et al*., 2000). Furthermore, cyclophilins have been identified as one of the virulence factors in plant pathogenic fungi in a number of studies (Viaud *et al*., 2002; Harel *et al*., 2006; Gan *et al*., 2009).

The objective of this study was to isolate the *cyp* cDNA sequence from *G. boninense*. This article reports on the isolation of a partial *cyp* cDNA sequence from *G. boninense*. The isolated fragment has been analysed by homology searching in the Genbank database, and has been aligned with the plant pathogenic fungus cyclophilin sequences to confirm the identity of the sequence.

MATERIALS AND METHODS

Ganoderma boninense Culture Preparation

The *G. boninense* culture was obtained from the stock collection of the Plant Pathology and Weed Science Group (PPWS) of the Malaysian Palm Oil Board (MPOB), Bandar Baru Bangi, Selangor. The culture was grown on a potato dextrose agar (PDA) plate for 14 days prior to RNA extraction.

Total RNA Extraction

The mycelia was scraped from the PDA agar surface and quickly frozen with liquid nitrogen before being ground into powder. Total RNA was isolated from the mycelial tissues of *G. boninense* using the RNeasy® Mini Kit (Qiagen, Germany). The RNA quality and quantity were determined using the Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc, USA).

Identification and Primer Design for *cyp* Gene

A few pathogenic fungus *cyp* genes were identified from the GenBank database. The amino acid and DNA sequences of these genes were aligned using the Vector NTI software (Invitrogen, USA). Conserve sequences were identified, and two pairs of degenerate primers were designed based on these sequences. They were forward primers, CYP1 5′-ACTTYATGCTYCAGGGYGGTG-3′ and CYP3 5′-AACGGYACYGGYGGCAAGTC-3′, and reverse primers, CYP2 5′-AASACRACGTGCTTGCCGTC-3′ and CYP4 5′-CCGTRATGAAGAACTGCGARC-3′. The *cyp* sequences used in primer design were from *Brotryotiana fuckeliana* (XM_001559534), *Gibberella zeae* PH-1 (XM-387615), *Magnaporthe grisea* (AB062457), *Phaeosphaeria nodorum* SN15 (XM_001802880) and *Sclerotinia sclerotiorum* 1980 (XP_001593362).

First-strand cDNA Construction and Amplification of Partial-length *cyp* cDNA

First-strand cDNA was synthesised from the total RNA isolated from *G. boninense* mycelia using the SMART™ RACE cDNA Amplification Kit (Clontech, USA). PCR amplifications were carried out using different combinations of primers (combination of forward primers, CYP1 & CYP 3, and reverse primers, CYP2 & CYP4) which flank in the middle region of the gene. PCR amplifications were carried out using the AccuPrime™ *Taq* DNA Polymerase System (Invitrogen, USA). The PCR mixtures were prepared in a total volume of 50 µl, containing 2.0 µl of cDNA (185.6 ng μl^{-1} for 5′ cDNA and 139.9 ng μl^{-1} for 3′ cDNA), 2.0 µl of forward primer (10 µM), 2.0 µl of reverse primer (10 µM), 5.0 µl of 10X AccuPrime™ PCR Buffer I and 1.0 µl of AccuPrime™ *Taq* DNA polymerase. The thermocycler was programmed for 1 min pre-heating at 94°C, followed by 35 cycles consisting of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 68°C for 2 min; then, a final extension for 5 min at 68°C.

DNA Cloning

The PCR products were separated by 1% agarose gel electrophoresis. PCR products with a single fragment of size about 200 bp were selected for cloning. The DNA fragments were then extracted from the agarose gel using the QIAquick® Gel Extraction Kit (Qiagen, Germany). The purified fragments were ligated into the pCRII-Topo vector (Invitrogen, USA) and transformed into *Escherichia coli* Strain DH5α. *Eco*RI digestion was carried out to confirm ligation of the insert.

DNA Sequence Analysis

Plasmid DNA of clones with the insert was prepared using the QIAprep® Miniprep Kit (Qiagen, Germany). The DNA sequence of representative clones was determined by sequencing on the Applied Biosystems 3730*xl* DNA Analyser. The DNA sequence analysis was carried out using the Vector NTI software (Invitrogen, USA). DNA and amino acid sequence homology searches were performed using BLAST 2.0 (Altschul *et al*., 1997) on the GenBank database. In addition, the conserved domain present in the sequence was determined.

RESULTS AND DISCUSSION

Isolation and Cloning of Partial Length *cyp* Gene

Total RNA of *G. boninense* was successfully isolated from its mycelia (*Figure 1a*), and the first strand cDNA was synthesised from the RNA using the SMART™ RACE cDNA Amplification Kit (Clontech, USA). The two pairs of forward and reverse primers were designed. PCR amplifications were carried out using different combinations of these primers. The forward primers, CYP1 and CYP3, were cross-combined with the reverse primers, CYP2 and CYP4, in the amplifications. The amplifications produced fragments with a size around 200 bp (*Figure 1b*). The amplicons were cloned into the pCRII-Topo vector (Invitrogen, USA) and transformed into *E. coli* Strain DH5α. Digestion of the representative clones using *Eco*R I was carried out, and all the clones were shown to contain the ~200 bp insert (*Figure 2*). Representative positive clones were then sequenced.

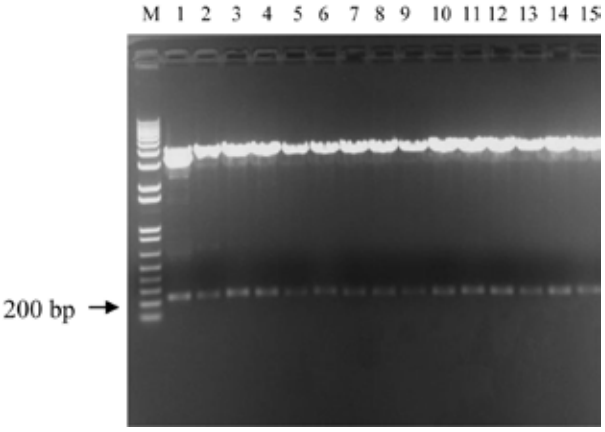

Figure 2. Eco*RI digestion of representative clones. Samples 1-5 were representative clones from clone Gc3, samples 6-10 were representative clones from clone Gc5, and samples 11-15 were representative clones from clone Gc7. Electrophoresis was carried out in 1.0% agarose gel. M = 1 kb Plus DNA Ladder (Invitrogen, USA).*

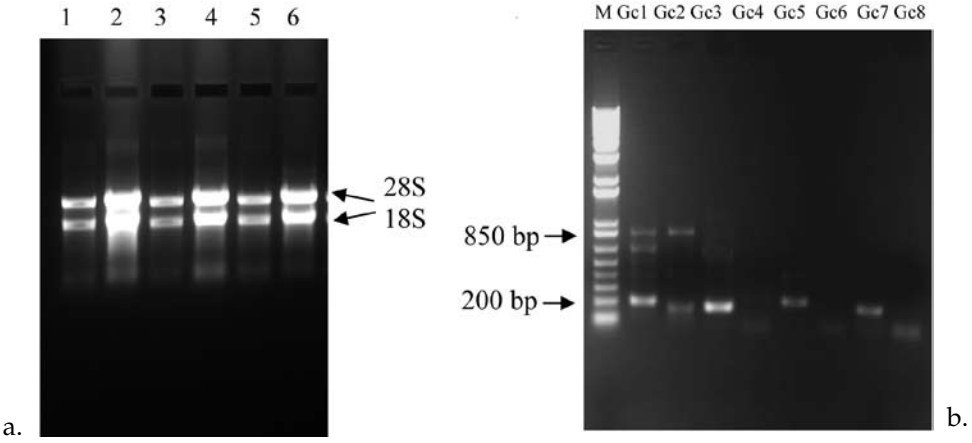

Figure 1. a. Electrophoresis of Ganoderma *total RNA (lane 1-6) in 1.0% agarose-formaldehyde gel. b. PCR amplification using combinations of two degenerate primers. Combinations of primers: Gc1 = CYP1, CYP2; Gc2 = CYP1,CYP4; Gc3 = CYP3, CYP2; Gc4 = CYP3, CYP4; Gc5 = CYP1, CYP2; Gc6 = CYP1,CYP4; Gc7 = CYP3, CYP2; Gc8 = CYP3, CYP4. Samples 1-4 used 5′ cDNA as template while samples 5-8 used 3′cDNA as template. Combinations No. 3, 5 and 7 successfully amplified a single fragment (~200 bp). Electrophoresis was carried out in 1.0% agarose gel. M = 1 kb Plus DNA Ladder (Invitrogen, USA).*

DNA Sequence Analysis

DNA sequencing results show that all the amplified fragments contained the same DNA sequence. However, clone Gc5 was shown to contain the longest sequence which was 212 bp. Results of the BLAST homology search show that this DNA sequence was highly similar to other fungal *cyp* gene sequences. The identity was in the range of 78%-82% (*Table 1*). Similarly, the translated amino acid sequence of the clone was highly similar to other plant pathogenic fungal CYP, such as *B. fuckeliana*, *G. zeae* PH-1, *M. grisea*, *P. nodorum* SN15 and *S. sclerotiorum* 1980 (*Figure 3*).

Besides that, the conserved domain that was possibly present in the partial sequence was determined. The search was performed using the Conserve Domain Database (CDD) software available at http://www.ncbi.nlm.nih.gov/cdd (Marchler-Bauer *et al*., 2005). The search result shows that the obtained sequence carries a cyclophilin_ABH-like domain. This domain contains the peptidylprolyl *cis-trans* isomerase (PPIase) domain. PPIase is an enzyme having a function in accelerating protein-folding by catalysing the *cis-trans* isomerization of the peptide bonds preceding proline residues. This enzyme has been implicated in protein-folding processes which depend on catalytic/chaperone-like activities.

Cyclophilins are a highly conserved family of proteins in bacteria, fungi, plants and animals. Besides being implicated in various cellular

TABLE 1. SUMMARY OF BLAST RESULTS FOR CLONE DNA SEQUENCE

Genbank accession	Description	Identity %
AB231808.1	*Malassezia dermatis* CYP mRNA for cyclophilin, partial cds	82
XM_387615.1	*Gibberella zeae* PH-1 hypothetical protein partial mRNA	81
XP_001593362	*Sclerotinia sclerotiorum* 1980 peptidyl-prolyl *cis-trans* isomerase (SS1G_06284) partial mRNA	80
XM_001559534.1	*Botryotinia fuckeliana* B05.10 peptidyl-prolyl *cis-trans* isomerase (BC1G_01740) partial mRNA	80
AL116652.1	*Botrytis cinerea* strain T4 cDNA library	80
XM_568801.1	*Cryptococcus neoformans* var. neoformans JEC21 cyclophilin A (CNB01290) partial mRNA	78
AB062457.1	*Magnaporthe grisea* CYP1 mRNA for cyclophilin, complete cds	78
XM_754780.1	*Ustilago maydis* 521 hypothetical protein (UM03726.1) partial mRNA	78

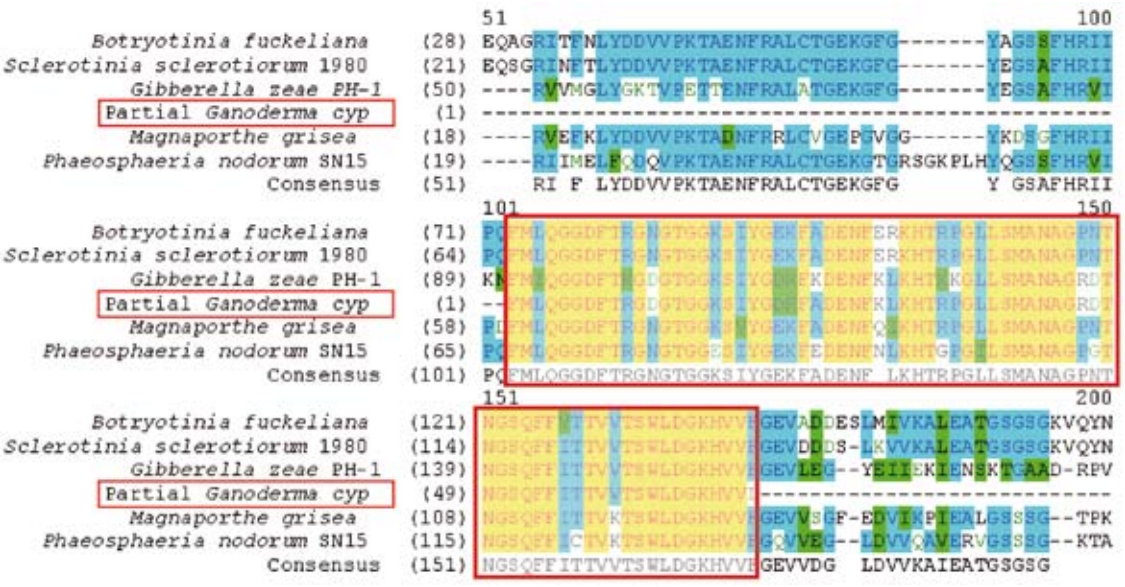

Figure 3. Alignment of translated amino acid sequence with other plant pathogenic fungal cyclophilin. Highly conserved sequences are highlighted in yellow. Amino acid sequences boxed in red lines indicate high similarity to cyclophilin_ABH_like domain.

processes, cyclophilins have also been suggested to be involved in fungal pathogenicity. Downregulation of the gene has affected fungal appresoria, and leads to a reduction in fungal virulence. For example, deletion of *cyp-1* in the pathogenic fungus *M. grisea*, a causal agent of rice blast, resulted in a lower rate of infection but did not affect the vegetative growth of the fungus in culture (Viaud *et al.*, 2002). The deletion also affected the function of the appressoria, which caused the fungus to lose its ability to penetrate the plant cuticle efficiently. Thus, it seems that *cyp* gene activation is essential for *M. grisea* pathogenicity. In addition, cyclophilins are also required for virulence in the human pathogenic fungus, *Cyptococcus neoformans* (Wang *et al.*, 2001).

The availability of the partial sequence may facilitate isolation of the full length sequence of the *G. boninense cyp* gene. This will allow studies on the *cyp* gene function and regulation in *G. boninense* in the future. Studies on regulation of the *cyp* gene may provide clues on how the gene is being regulated during invasion of the oil palm host by *G. boninense*.

CONCLUSION

A 212 bp partial length of *cyp* gene has been successfully isolated from *G. boninense*. The cloned fragment has been shown to have high similarity to other plant pathogenicity fungal *cyp* genes. Isolation of the full-length clone will be carried out to establish the role of the *cyp* gene in *G. boninense* pathogenicity.

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