SHORT COMMUNICATION

Journal of Oil Palm Research Vol. 26 (3) September 2014 p. 265-269

ISOLATION OF A PARTIAL cDNA CLONE CODING FOR *Ganoderma boninense pde*

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ABSTRACT

Ganoderma *is white rot fungus that causes a major disease in oil palm. Control measures that have been introduced have not been able to curb the disease effectively. Efforts are underway to uncover more information on the* Ganoderma-*oil palm interaction, especially at the molecular level. During plant infection, a few genes are highly expressed in fungus and have been suggested to be related or involved in fungal pathogenicity. One of these genes is the phospholipid-transporting ATPase (*pde*). In* Magnaporthe grisea*, its protein has been suggested to be associated with hyphae development and proliferation. Degenerate primers were designed based on the conserved regions of the gene. These primers were successfully used to amplify a 544 bp cDNA fragment from* Ganoderma boninense*. DNA sequence analysis showed the fragment has about 80% similarity to other plant pathogenic fungus* pde *genes.*

Keywords: *Ganoderma boninense*, pathogenicity, basal stem rot, phospholipid transporting ATPase, cDNA.

Date received: 25 September 2013; **Sent for revision:** 30 September 2013; **Received in final form:** 14 July 2014; **Accepted:** 16 July 2014.

INTRODUCTION

Fungi cause more plant diseases than any other group of microbes (Lu *et al*., 2003). *Ganoderma*, a basidiomycete, has long been identified as a 'white rot' pathogen (Paterson, 2007) that causes basal stem rot (BSR) disease in oil palm. BSR is a major oil palm disease that threatens the oil palm plantation industries in Malaysia and South-east Asia and warrants urgent attention. The disease has caused huge economic losses through direct reduction in the number of standing palms as well as in bunch number and weight. The disease can result in up to 80% death of standing palms which are only half-way through their economic life (Singh, 1991). At present, the disease is believed to be primarily caused by *Ganoderma boninense*. Although there are at least three other species that have been known to infect oil palm, namely *G. zonatum, G. miniatocinctum* and *G. tornatum*, *G. boninense* is considered to be the most aggressive fungus towards oil palm (Idris *et al*., 2001).

For the past several years, attempts have been made by the industries and researchers to improvise procedures to control *Ganoderma* in order to curb the economic losses. Procedures include surgery and soil mounding, application of fungicides, removal of *Ganoderma* infected palms and appropriate techniques for replanting (Idris *et al*., 2004a, b; 2005; Chong and Mohd Ahdly, 2007). Efforts have also been seen in the field of biological control of *Ganoderma*. Studies on antagonistic fungi, such as *Trichoderma* and *Arbascular mycorrhiza*, have indicated some promising results in controlling *Ganoderma* (Sariah, 2003; Nurrashyeda *et al*., 2011). These fungi limit the growth of *Ganoderma* by competing for resources. Efforts are also under way to develop oil palm progenies that are tolerant towards *Ganoderma*. A few of these screening efforts have resulted in the identification of tolerant palms (Idris *et al*., 2004c; Breton *et al*., 2009). Equally important, efforts have also been made to formulate procedures for early

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detection of *Ganoderma* infection in oil palm. These include the procedures with PCR-based detection and ELISA techniques (Kandan *et al*., 2009; Idris *et al*., 2003). Besides these efforts, no single treatment has effectively tackled the problem.

More recently, efforts have been made to gain more understanding on *Ganoderma*-oil palm interaction, especially at the molecular level. The aims are to discover the genetic factors that contribute to pathogenicity. It is hoped that more effective strategies against *Ganoderma* could be formulated once the interaction is better understood. Studies on the gene expression and protein profiles of infected and non-infected palms have been on-going at the Malaysian Palm Oil Board (MPOB). To date, a number of putative genes and phenolic compounds that may be related to plant defense system have been identified (Mohamad Arif *et al*., 2007). Efforts have also been made to try to understand the fungus itself. This has led to recent *Ganoderma* genome sequencing efforts by MPOB. The complete drafts of *Ganoderma* genome sequences were announced in early 2011 (http://www.mpob.gov.my). In this work, we were interested to study genes that have been suggested or indicated to be related to *Ganoderma* pathogenicity. This is believed to be a more straightforward way to obtain genes that potentially play important roles in *Ganoderma* infectivity. These genes are potentially useful in efforts to understand the *Ganoderma*-oil palm interaction and ultimately for controlling the disease. In this work, we aimed at isolating and characterising a number of these genes including cyclophilin (Lim *et al*., 2011), laccase (unpublishded data), cAMP-dependent protein kinase (PKA) (unpublished data) and phospholipid transporting ATPase (*pde*). Here, we report the isolation of a partial length cDNA coding for *pde*. The availability of the gene sequence will hopefully facilitate understanding of the infection mechanism of the fungus.

MATERIALS AND METHODS

Fungi Growth and Maintenance

G. boninense culture (PER71) was obtained from the stock collection of the *Ganoderma* and Disease Research for Oil Palm (GANODROP) Unit, MPOB. The culture was grown on potato dextrose agar (PDA) plate for 14 days before being used for RNA extraction.

Isolation of Total RNA and cDNA Synthesis

The mycelium of *G. boninense* was scraped from the PDA agar surface and quickly frozen with liquid nitrogen before being ground into powder. Total RNA was isolated from the mycelium tissues. Total RNA extraction was carried out using RNeasy® Mini Kit (QIAGEN). RNA quality and quantity were determined using Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc.). First-strand cDNA was synthesised from 1 ug of total RNA using SMART™ RACE cDNA Amplification Kit (Clontech) according to the manufacturer's protocol.

RTPCR Amplification and Cloning

Oligonucleotide primers used in this study were synthesised based on the conserved regions of fungal *pde* genes. Fungal *pde* sequences available at the GenBank were retrieved and aligned for the identification of the conserved regions. The fungal *pde* cDNAs that were used in the sequence alignment for the identification of possible conserved blocks were from *Magnaporthe grisea* (GenBank Accession # AY026257), *Postia placenta* (GenBank Accession # XM002473961), *Schizophyllum commune* (GenBank Accession # XM003035634), *Coprinonsis cinerea* (GenBank Accession # XM001841129) and *Ustilago maydis* (GenBank Accession # XM754284). The fidelity of these identified conserved regions was evaluated using BLAST search at the Genbank databases. Only regions with good fidelity were used for the primer synthesis.

RTPCR was carried out using the Advantage 2 Kit (BD Biosciences) with 35 sequential cycles at 94°C for 30 s, 58°C for 30 s and 72°C for 3 min on PTC-100 Programmable Thermal Controller (MJ Research). The PCR products were electrophoresed, and the amplified bands were excised and purified using QIAEXII Gel Extraction Kit (QIAGEN) and finally ligated into PCR®II-TOPO® vector (Invitrogen). Plasmid DNA from several selected colonies was isolated using QIAGEN Plasmid Preparation kit (QIAGEN) according to the manufacturer's protocol. Confirmation of insert was performed with *EcoRI* digestion and followed by agarose gel electrophoresis.

DNA Sequence Analysis

Plasmid DNA for sequencing was prepared using QIAGEN miniprep Kit (QIAGEN). Analysis of DNA sequences was carried out using VectorNTI software (Invitrogen). The analysis included the removal of unreadable and vector sequences, sequence alignment, ORF identification and contig analysis and assembly. DNA and protein homology search against the GenBank databases was performed using BLAST 2.0 (Altschul *et al.*, 1997).

RESULTS AND DISCUSSION

RT-PCR and Cloning of Partial Length cDNA Encoding *Ganoderma pde* Gene

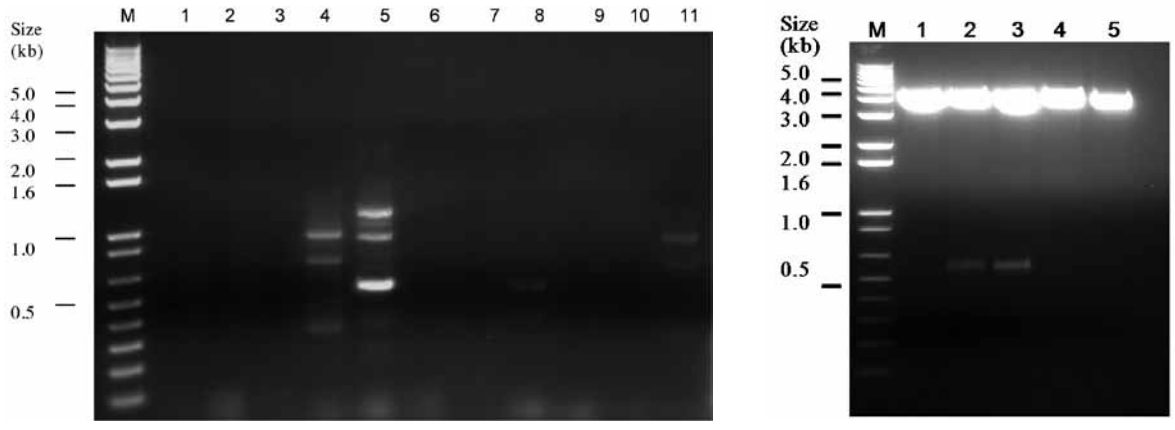
During the early process of fungal infection, fungi synthesise many compounds necessary for recognition, adhesion and penetration into plant tissues. In *M. grisea*, PDE has been suggested to be important for development of penetration hyphae and subsequent proliferation of the fungus inside the host tissues. The gene is expressed in germinating conidia and developing appresoria. A *pde1* mutant *M. grisea* was shown to be impaired in its ability to elaborate penetration hyphae (Balhadere and Talbot, 2001). Based on the expression pattern, the gene product was believed to be important for development of penetration hyphae and subsequent proliferation of the fungus beyond colonisation of the first epidermal cell.

Since the information of *Ganoderma pde* was not available at the onset of the work, the RTPCR work to isolate the cDNA was based on the utilisation of degenerate primers. These degenerate primers were designed based on *pde* sequences from other fungi available at the GenBank database (http://www.ncbi.nlm.nih.gov/). The technique has been reliable for isolating genes with no sequence information. In our work, we have successfully isolated a few genes from oil palm and *Ganoderma* using the technique (Lim *et al*., 2011; Rasid *et al*., 2008; 2007). Our analysis of the fungus *pde* sequences resulted in the generation of six degenerated primers. The two sense primers are PDE11 (5′- TYT CCG YGA CMG GCA CKC TAA -3′) and PDE13 (5′- GAY GAA GCT GCK YTY GTK GCW - 3′) while the antisense primers are PDE12 (5′- ARA CSC MRG GAG ARA CRG GAC ARC - 3′) and PDE14 (5′- AGC TTG TCY TCM ARY GCM GTS GC - 3′).

One of the primer combinations (PDE10/13) was successfully optimised to produce a major amplified fragment of about 0.5 kb (*Figure 1*). Other primer combinations either produced no amplified product or several faint bands indicating unspecific binding. The amplified fragment was purified and cloned into PCR®II-TOPO® vector. Clones with inserts have been obtained and confirmed by *EcoRI* digestion (*Figure 1b*). The clone was designated as GPDE6.

DNA Sequence Analysis

The complete DNA sequence was obtained for representative clones originally from RT-PCR products. The sequence analysis results showed that the fragment size was 544 bp. The identity of the sequence was established using homology BLAST search to the Genbank database. Results from BLAST search indicated that the DNA sequence of GPDE6 was highly identical to *pde* sequences from other fungi at around 70% identity. The identity of translated amino acid sequence of the GPDE6 was examined using BLASTX. It was shown that the clone codes for 181 amino acid residues. The results also showed that the sequence shared a very high level of identity to PDE from other fungi including *Dichomitus squalens* (accession number XP 007361589), *Trametes versicolor* (accession number XP 008045338), *Fomitopsis pinicola* (accession number EPS98966), *P. placenta* (accession number XP 002474006) and *Phanerocheate carnosa* (accession number XP 007395378) with about 88%, 83%, 74%, 72% and 71%, respectively (*Figure 2*). The high identity of GPDE6 to other PDE sequences proves



(a) (b)

Figure 1. a) Electrophoresis of RTPCR amplification products of Ganoderma pde *gene using degenerate oligonucleotide primers. One of the primer combinations produced a major amplified product (Lane 5). b) Electrophoresis of representative clones obtained from the transformation of amplified fragments for* pde*. The plasmid DNA was digested with* EcoR I *to verify the presence of insert. Some of the clones were shown to carry the ligated insert (Lane 2 and 3). M is 1 kb Plus DNA Ladder.*

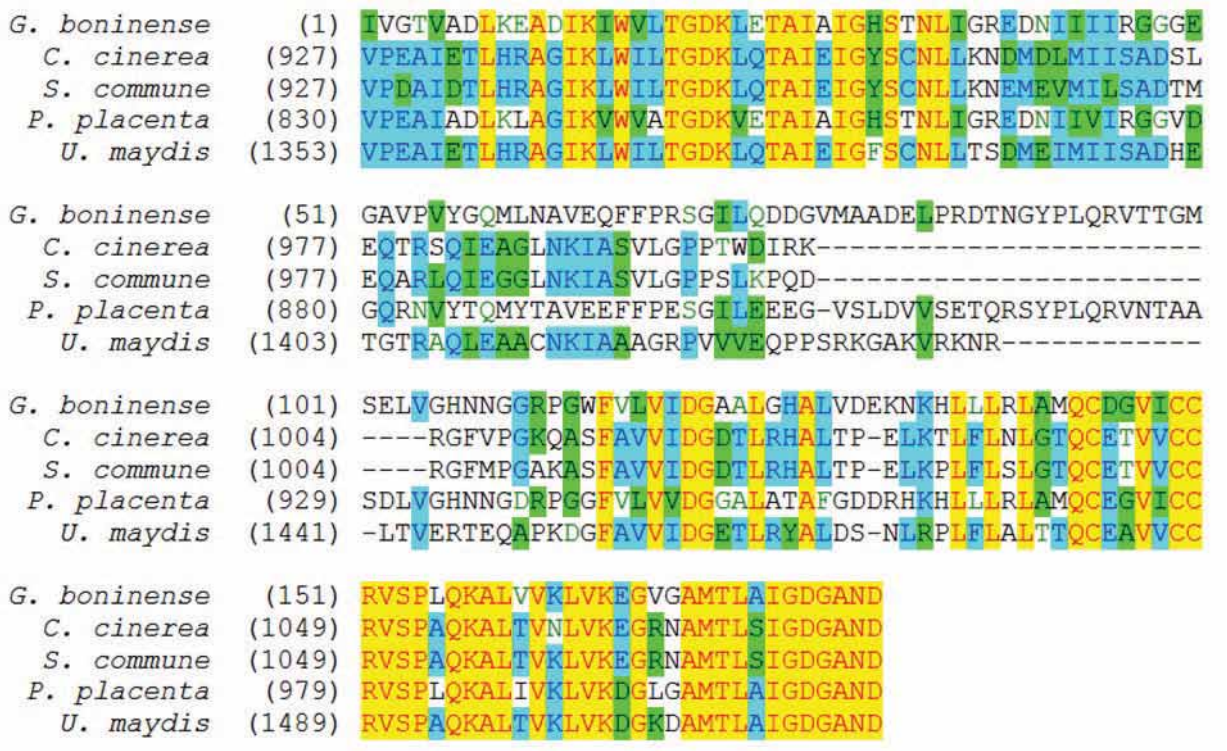


Figure 2. Comparison of deduced amino acid sequence of G. boninense *PDE to representative PDE sequences from other fungi. Yellow colour indicates exact match; blue colour denotes greater than 50% match; and green indicated weak similarity.*

that the clone indeed codes for *Ganoderma pde*. The results of conserved domains search performed using CDD software available at http://www.ncbi.nlm.nih.gov/BLAST (Marchler-Bauer *et al.*, 2009) showed the sequence contains a highly conserved domain, phospholipid-translocating P-type ATPase. This domain is responsible for transporting phospholipids across bilayer membranes.

CONCLUSION

This article reports the isolation of a gene that codes for one of the proteins involved in development and proliferation of *Ganoderma* hyphae. We have isolated and completely sequenced partial cDNA clones coding for PDE. Based on the sequence comparison either at the nucleic acid or deduced amino acid level, we concluded that the clones that we obtained indeed code for *G. boninense pde* as evidenced by their high identity to other fungal *pde* genes. Conserved aspartate-rich domains that are essential for catalytic activity were also observed within the sequence. The isolation of full-length clone will be carried to enable the study on the regulation and functional analysis of the gene.

ACKNOWLEDGEMENT

The authors would like to thank the Director-General of MPOB for permission to publish this article.

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