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Molecular isolation and characterization of the *Rac GTPase* gene from *Oryza sativa indica* cultivar UKMRC9

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Abstract

Rac GTPase is involved in the transduction and regulation of various cellular processes in eukaryotes such as the plant defense system. This regulatory gene has been reported to produce a broad spectrum resistance against pathogens in plants. The purpose of this study is to isolate and characterize the *Rac GTPase* gene from UKMRC9, a locally developed cultivar that shows tolerance to major rice diseases in Malaysia. Total RNA of infected young rice leaves with low disease index was extracted, reverse transcribed into cDNA and sequenced. The nucleotide sequence was translated into a 307 amino acid Rac GTPase protein and was subjected to *in silico* analysis to defer protein characteristics, evolutionary relations and structural characteristics. This protein was found to be 99% similar to the Rho GTPase in *Oryza sativa* and contains actin and actin-like protein domains that have been implicated in cytoskeletal modelling and remodelling in cells with a function in structure and primary defence against host infiltration. The 3D structure of the protein contains a 6 β- sheet structure that is surrounded by helixes, with an insert region and two switches that are characteristic of ATP binding Rac/Rho proteins. As Rac/Rho is diverse in plants, this isolated gene will be used in future mutational and transgenic studies that will enable us to elucidate the functional role of this protein in rice and compare the observed function with the reported functions observed in plants as well as other eukaryotic organism.

Keywords: *Rac, Rho*, Actin, defense, rice, ATP binding.
Abbreaviations: PDBs_Protein databases; UKMRC9_Universiti Kebangsaan Malaysia Rice Cultivar 9; *GTPase_Guanosine Triphosphatase.*

Introduction

Resistance to penetration of the plant epidermis is the first line of defense that must be overcome by most microorganisms. Plant cytoskeleton consisting of actin filaments and microtubules plays an important role in the stabilization of host cell polarity during plant growth and defense. The restructuring of the actin cytoskeleton in plant-pathogen interaction results in the translocation of cytoplasm and nucleus to the site of penetration (Fujiwara et al., 2006).

The *Rac GTPase* gene has been implicated in the manipulation of cytoskeleton restructuring which is essential in defense against pathogen penetration (Kawasaki et al., 1999). Sakumura et al. (2005) proposed a model which helps to explain the activity of Rhos as well as their relationship to motion. Three proteins such as Cdc42, RhoA, and Rac are encompassed in this model. The Cdc42 was assumed to encourage filopodia elongation and block actin depolymerization. The RhoA was considered to encourage actin retraction. The Rac GTPase was further divided into four subgroups including Rac1, Rac2, Rac3 and RhoG. The small GTPase superfamily is divided into at least five families, including Ras, Rho, Rab, Arf, and Ran (Takai et al., 2001). Plants seem to lack Ras family of small GTPases, which has a crucial signaling role in growth and development of animals and most other eukaryotes. However, plants have a large number of Rho-like proteins termed Rop (Rho related GTPase from plant) or Rac (OsRac for rice Rac) (Yang, 2002; Zeng and Yang, 2000), which are highly conserved in the plant kingdom. The identification of cellular function for specific genes in the *Rac GTPase* family especially in *Oryza sativa* is largely unknown (Nguyen et al., 2007) and complex as there is more than one member involved in the process of transduction.

Rice (*Oryza sativa*) contains about seven *OsRac* genes (Kawasaki et al., 1999; Miki et al., 2005) and all genes are expressed in seeds, leaves, stem and root. However, *OsRac*2, *OsRac*6 and *OsRac*7 are expressed at very low levels in leaf blades (Chen et al., 2010). However, this group of genes seem to be more complex in *Arabidopsis thaliana*, where it has eleven genes (Yang, 2002; Miki et al., 2005). The presence of this gene in both monocots and dicots suggests that this gene is conserved in both plant groups (Miki et al., 2005). According to Ridley (2006), *Rac* is a subfamily of the Rho family of GTPases and is an approximately 21 kDa signaling G proteins that binds to immune receptors downstream and triggers innate immunity in rice, barley and other species (Kawano et al., 2010).

Although *Rho GTPases* were shown to be involved in the regulation and organization of the actin cytoskeleton and cell polarity development in eukaryotes (Fu et al., 2002) it was shown later that *Rho* signaling controls various processes in plants such as cell wall synthesis, H_2O_2 production, cell elongation and cell differentiation and regulation of

hormones in various eukaryotic organisms (Ridley, 2000). The *Rho* was known to have cytoskeletal effects on stress fiber and focal adhesion. However, little is known on the genomic structure of the members of the *Rho GTPase* family in plants and how they actually evolved through time (Winge et al., 2000).

In rice, OsRac1, which is located in the plasma membrane, is involved in reactive oxygen species (ROS) production through an NADPH oxidase, and in the initiation of cell death during defense signaling (Kawasaki et al., 1999; Ono et al., 2001). The *OsRac1* functions as a positive regulator of NADPH oxidase activation in PAMP signaling (Ono et al., 2001), and in suppression of expression of scavenger metallothionein (Wong et al., 2004) in transient accumulation of ROS. Expression of CA-OsRac1 enhances resistance to rice blast and bacterial blight infections (Ono et al., 2001, Suharsono et al., 2002), whereas DN-OsRac1 suppresses HR induced by incompatible races of rice blast fungus, indicating that *OsRac1* is one of the key regulators of rice innate immunity (Ono et al., 2001). The *OsRac1* regulates the stability and activation of OsMAPK6, a rice mitogen-activated protein (MAP) kinase, by sphingolipid elicitors (Lieberherr et al., 2005).

Although the physiological functions of several of these GTPases or their associated proteins have been examined in plants, much remains unknown and requirs investigation due to the diversity within the Rac and the functions that are moderated by Rac GTPase. Although the *Rac* has been studied in *Oryza sativa*, no research has been done on the indica type of rice species. Here, we report the isolation and molecular characterization of the *Rac GTPase* UKM gene in some selected Malaysian rices. We hope that the information derived from this analysis will provide us with sufficient information to direct our functional and trangenics studies.

Results and Discussion

Infection of Oryza sp. with Rhizoctonia solani

Four rice varieties (MR219, UKMRC2, UKMRC9 and *Oryza rufipogon*) were screened for their response to the *Rhizoctonia solani* strain 1802. The disease severity index (DSI) of these four varieties was monitored over 10 days post inoculation (Dpi) (Table 1). According to previous studies involving different rice varieties and comprehensive screening for resistance in rice plants against *R. solani,* no full resistance against infections was found (Lee et al., 2004). The infection can spread and infect the plant completely when it is under optimal conditions, low light intensity, high humidity and low temperatures (Eizenga et al., 2002). The disease severity, pathogenicity and virulence vary from plant to plant species.

Table 1. Disease severity index of rice varieties screened with *Rhizoctonia solani* 1802.

Variety	10 Dpi
O.rufipogon	2
MR219	3
UKMRC2	2
UKMRC9	1

Legend: Scale 1: below 20% of plant showing disease sympthoms; 2; below 40% of plant showing disease sympthom; 3, below 60% of plant shows disease sympthom; 4, 80% of plant diseased; 5, plant is completely disease or dead.

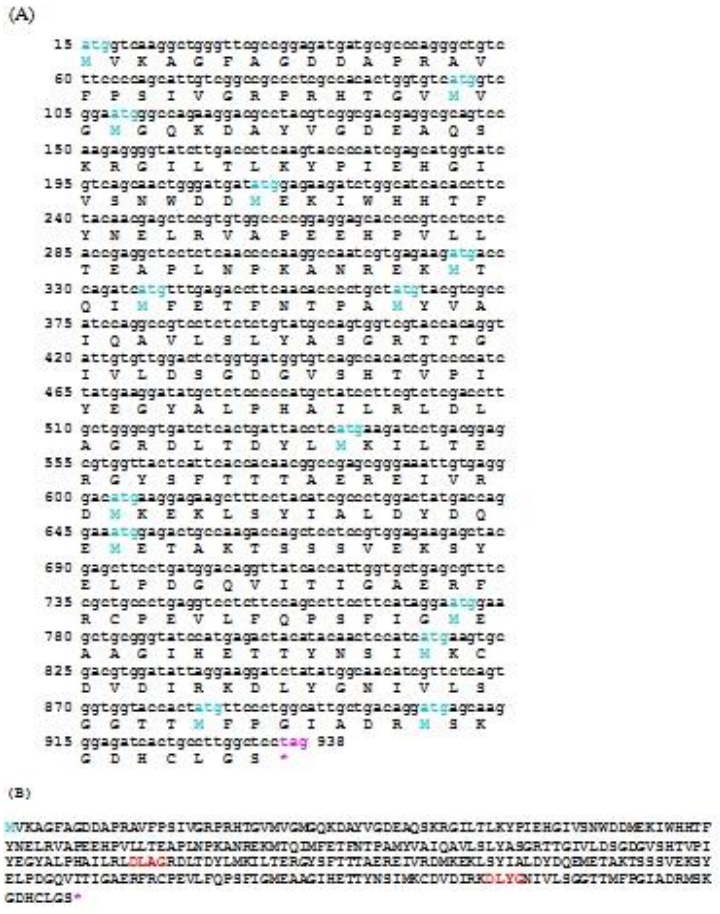

Fig 1. The Sequence of the *Rac GTPaseUKM* Gene from rice. (A) The ORF Finder analysis of the amplified sequence of the *Rac GTPase* gene. The sequence above is the nucleotide sequence while the sequence below is the amino acid. (B) The translated protein sequence of the *Rac GTPase* protein. The asterisk indicates the location of the STOP CODON. The residues in red are the Walker B motif.

The effects of *R. solani* on leguminous crops such as virulence and pathogenicity varies and have caused different levels of resistance and susceptibility in disease assays (Pascual et al., 2000). The differences in disease severity severity between varieties is displayed in Supplementary Fig.1.

Isolation of the Rac GTPase UKM gene

The amplified product sequence provides us with an ORF from nucleotide 15 (putative start codon) to 938 (putative stop codon) (Fig. 1A). Fig. 1B represents the 307 long amino acid of the Rac GTPase protein that was isolated from UKMRC9. The results from the ORF Finder analysis was blasted against the NCBI database and it returned 99% homology to *Oryza sativa* japonica *Rac GTPase* actin mRNA

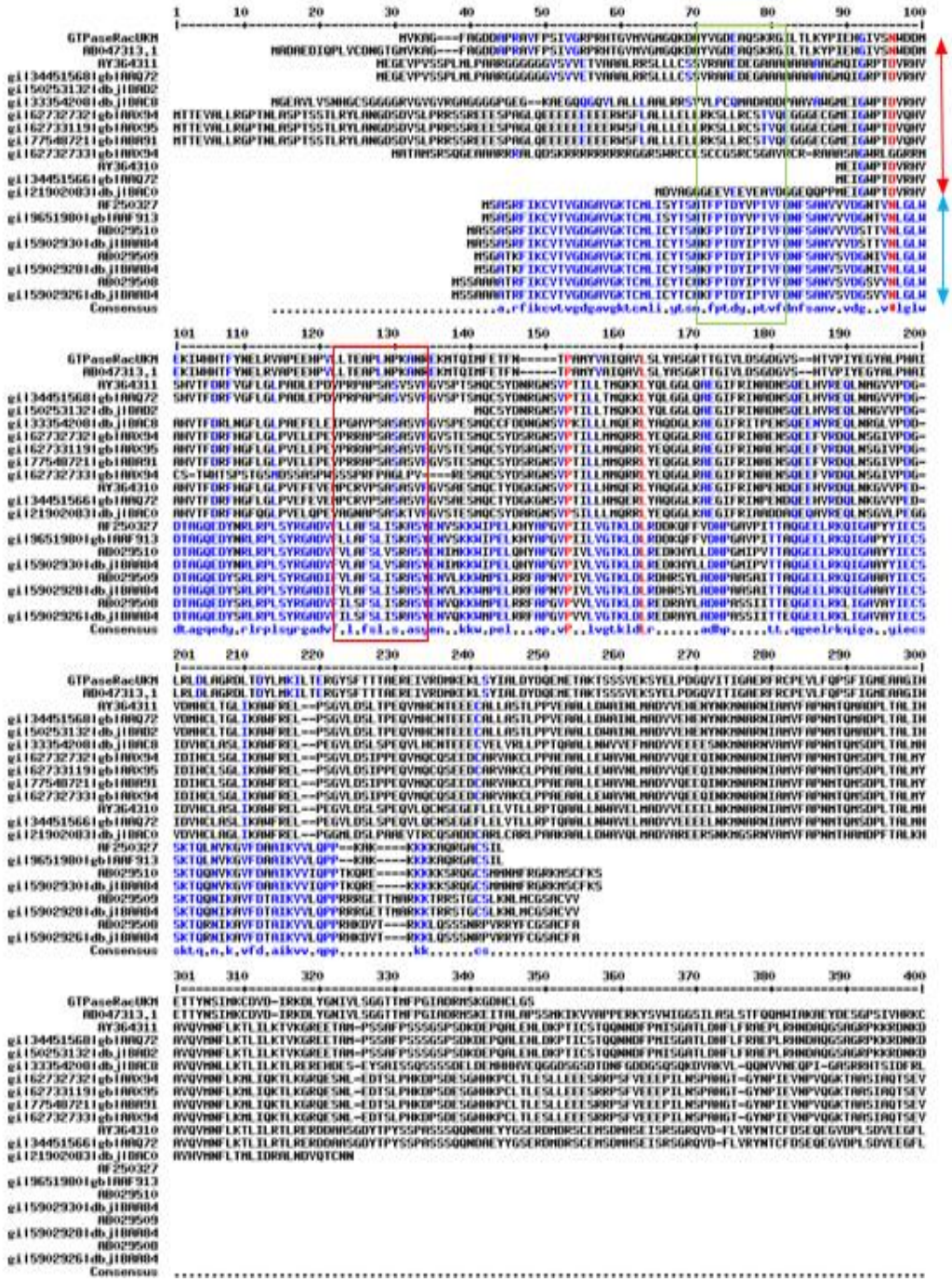

Fig 2. Multiple Sequence Alignment of *Rac GTPase* Protein. The regions of high homology are indicated by the consensus region sequences in blue. The red boxes in the alignment are the location of the motif/domains related to actin or actin-like function. Alignment identity of each sample: *GTPaseRac*UKM, AF250327 *Oryza sativa* small GTP-binding protein RACBP (RACB), AB029510 *Oryza sativa* mRNA for small GTP-binding protein *OsRac3*, AB029509 *Oryza sativa* mRNA for small GTP-binding protein *OsRac2*, AB029508 *Oryza sativa* mRNA for small GTP-binding protein *OsRac1*, AY364311 *Oryza sativa* (japonica cultivar-group) *Rho GTPase* activating protein 2, AY364310 *Oryza sativa* (japonica cultivar-group) *Rho GTPase* activating protein 1, AB047313.1 *Oryza sativa* japonica Group *OsRac1* mRNA for actin, BAA84492.1| small GTP-binding protein *OsRac1* [*Oryza sativa*], BAA84493.1| small GTP-binding protein *OsRac2* [*Oryza sativa*], BAA84494.1| small GTP-binding protein OsRac3 [*Oryza sativa*], AAF91343.1|AF250327_1 small GTP-binding protein RACBP [*Oryza sativa*], BAC05631.1| putative *rac GTPase* activating protein [*Oryza sativa* japonica Group], BAC81174.1| *Rac GTPase* activating protein 3 -like protein [*Oryza sativa* japonica Group], AAQ72347.1| *Rho GTPase* activating protein 1 [*Oryza sativa* japonica Group], AAQ72348.1| *Rho GTPase* activating protein 2 [*Oryza sativa* japonica Group], BAD29378.1| putative *Rho GTPase* activating protein 2 [*Oryza sativa* japonica Group], AAX94851.1| *Rac GTPase* activating protein 1 [*Oryza sativa* japonica Group], AAX94852.1| *Rac GTPase* activating protein 1 [*Oryza sativa* japonica Group], AAX95236.1| *Rac GTPase* activating protein 1 [*Oryza sativa* japonica Group], and ABA91518.1| *Rac GTPase* activating protein 1, putative, expressed [*Oryza sativa* japonica Group]. Green box: Actin 1 domain and Red Box: Actin Act like domain. Red and blue arrows indicating sequences with different levels of sequence similarity.

(AB047313.1) which is the template used in the primer design (Westwick et al., 1997; Li-zhen et al., 2002).

Characterization of Rac GTPase UKM protein

The ProtParam analysis showed that the molecular weight of the Rac GTPase UKM protein is 34.115 kDa and the calculated isoelectric point (pI) is 5.32, which indicates that this protein is acidic in nature. This result will be important for developing the buffer systems for purification of proteins by isoelectric focusing and two-dimensional electrophoresis (Krishna et al., 2011). The estimated molecular weight of this protein is similar to actin-like proteins and falls well within the molecular weight range of ~21 kDa predicted for this group of proteins (Boureux et al., 2007; Bustelo et al., 2007). The ProtParam program showed that the result for the Rac GTPase UKM protein is a net negative charge. The instability index is computed to be 32.73; thereby, classifying this protein as stable (Supplementary Fig. 2).

According to the TMHMM2.0 analysis of Rac GTPase UKM, the protein showed some inner and outer membrane activity and; therefore, it is likely that this protein is bound to the outer membrane of the cell (Supplementary Fig. 3). The data showed that amino acids 1 to 307 were located outside the cell membrane. Results of the SignalP 4.1 analysis demonstrated that there was no signal peptide present in this protein (Supplementary Fig. 4). The absence of a putative signal peptide indicates that this protein most likely remains intracellular (Krishna et al., 2011). The peptidase I cleavage site is determined from the Y score, where the S score is steep and C score is significant. Here, we observed that the C, Y and S scores are low and; therefore, the protein is unlikely to be a secretory protein. Membrane proteins and secretory proteins usually contain signal peptides, which is indicative of cleavage activity and ability to be secreted from the cell (Janda et al., 2010).

Multiple sequence alignment of the Rac GTPase protein

The Multialin analysis was conducted between 21 amino acid sequences of *Rac GTPase* proteins from *Oryza sativa* (Supplementary Fig. 5). The 21 aligned *Rac* sequences showed a high level of identity between amino acids within the consensus regions of the gene. The homologous regions of this proteins, which contains the consensus sequence text within the alignment, is in blue within the text (Fig. 2). The homologous region begins from amino acid 44 all through to amino acid 242 (Fig. 2). The results of the alignment shows that this gene is highly conserved within the genus and species of *Oryza sativa*. But varies at the C and N terminals of the gene. However, C- and N-terminal regions of Rac in plants have been reported to be quite divergent among different RopGAPs (Borg et al., 1999).

However from the multiple alignment we can observe that the Rac proteins have been separated into two distinct groups (indicated by arrow in red and blue). The separation of these proteins into two groups can also be seen in Fig. 3, where the sequences indicated with a red arrow falls within two sub-clades in a single clade, in which one sub-clade has the *O. sativa Rho* proteins and the other *O. sativa* GTPase activating protein. The same was observed with the group indicated by the blue arrow.

The Conserved Domain and Prosite analysis conducted on Rac GTPase UKM protein (Marchler-Bauer et al., 2009) identified actin-related protein domains, Actin 1, and Actin-like domain and NBD sugar kinase. The locations of these domains are as follows: (1) PS00406Actins_1Actins signature 1 located between amino acid 10-20 –motif YVGDEAQs.KRG and (2) PS01132Actins_Act_Like between amino acid 61-73-motif LLTEApLNPkaNR. The analysis conducted on the 21 proteins showed that the predominant domains were the *Rho, RhoGAP-CRIB* and the ACTIN domains (Table 2) that are central to the function and regulation of Rho or Rho like proteins. Rac GTPase UKM contains these three domains that are similar to the Rho domains and is likely to be involved in energy binding and signal transduction (Yang, 2002).

Rho GAPs has the invariant Arg (residue 205 for Rac GTPase UKM) which is required for GAP catalytic activity (Rittinger et al., 1997; Leonard et al., 1998; Scheffzek et al., 1998). The GAP-like domain is most similar to the p50 rhoGAP, which preferentially activates Cdc42 GTPase (Lancaster et al., 1994). It is interesting that all plant Rac/Rho contain CRIB motif found in several Cdc42/Rac effector proteins (Burbelo et al., 1995; Table 2 Fig. 2). None of the known animal and fungal GAPs contains this motif. The CRIB motif and the GAP domain is joined by another conserved region a consensus sequence for src homology domain 3-binding motifs, PxxxxPxxP or PxxPR (Ren et al., 1993). Actin participates in many important cellular processes, including muscle contraction, cell motility, cell division and cytokinesis, vesicle and organelle movement, cell signalling, the establishment and maintenance of cell junctions and cell shape. Many of these processes are mediated by extensive and intimate interactions of actin with cellular membranes (Doherty and McMahon, 2008). The production of actin is also key to the process of infection by some pathogenic microorganisms. The actin domain has been implicated in substrate binding with the actin superfamily. Substrate binding to superfamily members such as actin is associated with closure of catalytic site cleft. Their function is modulated by allosteric effectors, which may act on the cleft closure. In addition, two Walker B motifs (DXXG) were identified in the protein sequence (Fig. 1B). The function of this conserved residue is to bind directly or indirectly to a metal ion that is important in the catalysis of ATP binding activity (Sprang, 1997).

Phylogenetic analysis of the Rac GTPase proteins of Oryza sativa

The sequences used in the multiple alignment in Fig. 2 were then subjected to a Maximum Parsimony (MP) and Maximum likelihood (ML) analysis to derive a phylogenetic relationship between the studied sequences. Fig. 3 is the Maximum likelihood analysis conducted with the 21 amino acid sequences of the Rac proteins in *Oryza sativa*. The Maximum Likelihood analysis placed the Rac GTPase UKM protein in a monophyletic relationship with the Rho GTPase activating proteins of *Oryza sativa* (Fig. 3). Likewise, the most parsimonious tree in Fig. 4 displayed Rac GTPase UKM as either clustered together or in a neighboring clade to Rho GTPase proteins. Both programs indicate the relatedness between the Rac and Rho proteins in rice.

Table 2. Conserved Domain Analysis of RacGTPase UKM. Results obtained from Conserved Domain (NCBI) and Prosite (SIB).

DOMAIN	DESCRIPTION &FUNCTION
 Rho	**Description:** GTPase activator proteins towards Rho/Rac/Cdc42-like small GTPases. **GO process(GO:0007264):** small GTPase mediated signal transduction **GO component (GO:0005622):** intracellular **GO function(GO:0005525):** GTP binding
 RhoGAP CRIB	**Description RhoGAP:** GTPase activator proteins towards Rho/Rac/Cdc42-like small GTPases. **GO process(GO:0007165):** signal transduction **GO component(GO:0005622):** intracellular **Description CRIB**:Small domains that bind Cdc42p- and/or Rho-like small GTPases. Also known as the Cdc42/Rac interactive binding (CRIB). **GO function(GO:0005515):** protein binding
NBD_sugar-kinase_HSP70_actin ACTIN	**Description:** The functional activities of several members of the superfamily, including hexokinases, actin, and HSP70s, are modulated by allosteric effectors, which may act on the cleft closure. **GO Process(GO: 0005524):** ATP binding **GO function (GO:0030479):** cellular function of actin cortical patch

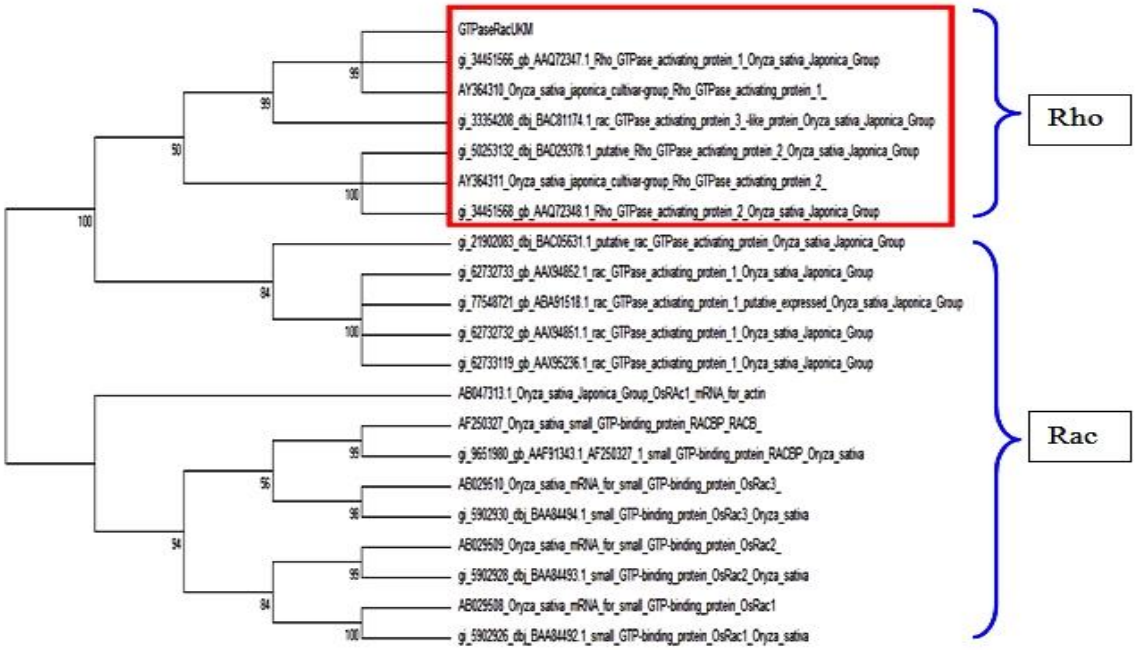

Fig 3. The evolutionary history of Rac GTPase proteins was inferred by using the Maximum Likelihood method based on the MEGA 5.10. The tree with the highest log likelihood is shown. The red box shows that Rac GTPase UKM was clustered together with Rho and other Rac family members. The phylogenetic tree exhibits that the twenty-one sequences could be clearly divided into two major clades which were then further divided into several subclades. Relationships between subclades within a clade are closer than those between clades.

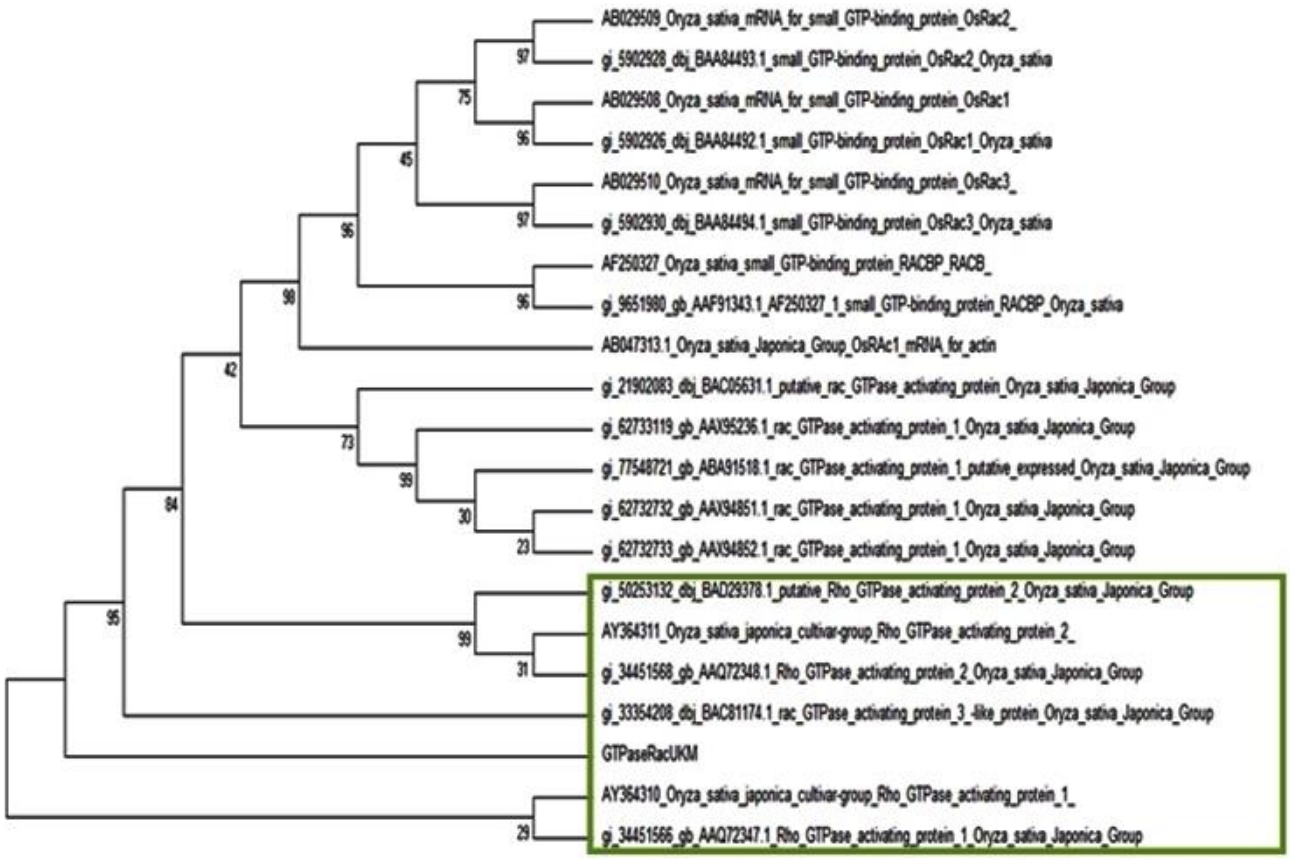

Fig 4. The evolutionary history of the Rac GTPase protein was inferred using the Maximum Parsimony method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees, in which the associated taxa is clustered together in the bootstrap test (1000 replicates), is shown next to the branches. Rac GTPase UKM is found within the green box clustered together with the most homologous protein sequences.

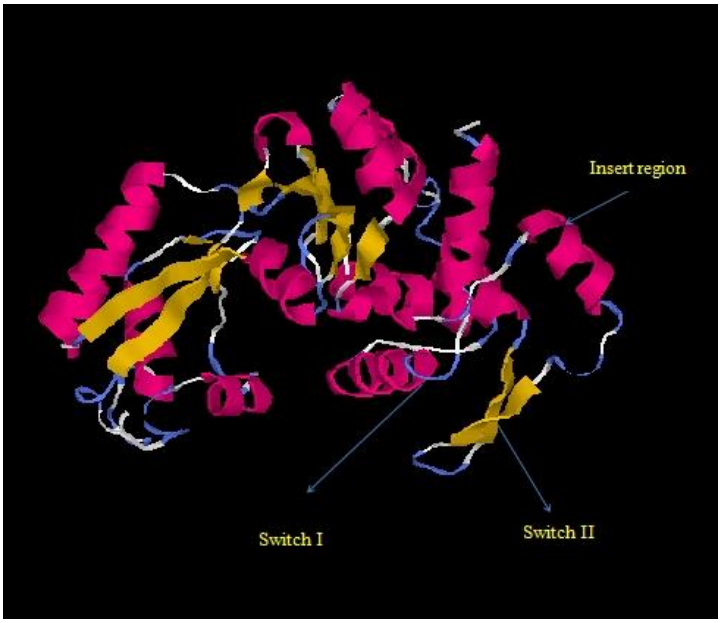

Fig 5. The 3D Structure of RacGTPase UKM modelled using the I-TASSER. The beta sheets are depicted in yellow while the helixes are in pink. The Rho/Rac interactive regions are indicated and labelled. The two switches and the insert regions are the active binding site of this protein. This is the region that is responsible for the binding of the protein into the DNA grooves.

The relatedness shown between the Rac and Rho GTPases can be explained by the process of diversification of Rho family GTPases, which is thought to be originated and evolved from an ancestral Rac-like GTPase (Van Gestel et al., 2002). The Rac-like GTPases found in plants are most likely from monophyletic origins, where an ancestral Rac-like gene is believed to have evolved into plant specific Rho-like GTPases (Duarte et al., 2006).

A large number of Rho-related GTPases have been found in plants since their initial identification in pea in 1993 (Winge et al., 2000). An evolutionary analysis of the plant Rho like GTPases suggests that they all belong to a unique subfamily named ROP (for Rho-related GTPase from plants) that form unique molecular switches involved in the regulation of actin organization and cell polarity as well as for the transmission of extracellular signals (Winge et al., 2000). Mutant analysis conducted in Arabidopsis implicated the role of F-actin in the mechanism underlying the ROP control of polar cell growth in different cell types, including tip-growing and non-tip growing cells (Jones et al., 2002). As seen in both the Maximum Likelihood and Maximum Parsimony trees generated in this study the Rac GTPase UKM protein is related to Rho GTPases and is highly homologous to *Oryza* actin-like proteins.

Protein structure analysis

The protein structure of *Rac GTPase* UKM was resolved using I-TASSER. Fig. 5 shows the 3D representation of the protein built by threading the alignment and structure of this protein against top 10 homologous protein structures available in databases. The PDBs used in this study are: 3eksA, 3dwlA, 1j6zA, 4eahD, 1yagA and 3qb0A. The developed model has a C score of 1.78, indicating that this structure has a very high level of confidence. Active sites were also predicted for this protein based on PDB3cjcA which has a C score ^{EC} 0.663. The active sites were predicted for 11-Gap, 26-Gap, 35-Gly, 37-Gap, 41-Gly, 45-Pro, 47-Ala, 49-Phe, 51-Ser and 79-Lys, while the predicted binding sites are 16-Gap, 26-Gap, 114-Val, 140-Ile, 143-Glu, 163-Leu, 164-Ser, 167-Ala and 171-Thr. Based on the domain results presented in Table 2, the predicted active binding sites lie within the domains that have been implicated in ATP binding activity. This is further supported by the gene ontology identity that provided a molecular function of ATP binding (GO: 0005524) and a cellular function of actin cortical patch (GO:0030479) activity for this particular protein.

In this study, it was essential to examine the already available *Rho GTPase* structures for prediction of a model for our protein. The three-dimensional structures of the human Rac1, Cdc42 and RhoA GTPases have been predicted through X-ray crystal analysis (Rudolph et al., 1999; Ihara et al., 1998). All these proteins consist of a central six-stranded β-sheet that is surrounded by loops and helixes, which form the surface of the protein. The Rac and Rho proteins have also regions that correspond to switch I and switch II of the Ras (Hirshberg et al., 1997). When the structures of the *Rac* and *Rho* proteins are compared, they are highly similar and the observed amino acid differences results mainly in different charge distribution across the surface of the proteins. The insert region, which is found in most members of the Rho family is a protruding amphipathic α-helix (Fig. 5). The amino acid composition of the insert helix varies between Rho and Rac/Cdc42 proteins. These small amino acid variations in the insert region may play an important role to differentiate between the various Rho-family members and their interacting proteins. The function of the insert region is still somewhat unclear and so far no proteins or protein motifs binding the insert region have been identified. Studies in human Rac proteins suggested that the insert region interacts with components of the NADPH oxidase (Joneson et al., 1998) and; thus, is key in ATP binding activity of the protein.

Materials and Methods

Source and varieties of plant materials

The rice varieties used in this study were *Oryza rufipogon* (IRGC105491), MR219 (Malaysian cultivated rice variety), UKMRC2 and UKMRC9. MR219 is susceptible to rice blast and sheath blight. *Oryza rufipogon* is a Malaysian wild rice veriety that has been reported to show resistance or tolerance to diseases and was used as a donor variety in the development of UKMRC2 and UKMRC9 with MR219 as recipient parent. (two lines developed through advance backcrosses between MR219 and *O. rufipogon*).

Fungal culture

The *Rhizoctonia solani* strain 1802 fungal culture was obtained from the paddy field in Kota Bharu, Kelantan, Malaysia. The fungal isolate was sub-cultured on potato dextrose agar (PDA) plates and stock cultures of this isolate were maintained on PDA slants. This particular fungal culture was used as it was obtained from our most recent sampling of diseased plots in Kota Bharu and showed comparatively more severe symptoms in rice compared to our other field isolates

Cultivation of plant materials

The four rice varieties; *Oryza rufipogon*, MR219, UKMRC2 and UKMRC9 were grown in 6 inch pots containing peat and clay soil in uncontrolled greenhouse conditions. A total of 24 seeds for each rice variety were planted and allowed to grow for 5-6 weeks before these plants were used in the disease severity test. Both parents of our own rice lines were included in this screen; so, that we are able to compare the DSI (Disease Severity Index) of these lines to their parents.

Preparation of fungal inoculum

R. solani 1802 cultures were sub-cultured on PDA and left for 5 days at room temperature for optimal growth in the dark. Mycelial plugs were prepared from the peripheral zones of the fungal mycelium cultures. A 5 mm fungal plug was placed at the sheath base of 5-6 weeks old plants (Park et al., 2008). A total of 8 rice plants for each variety were inoculated with *R. solani* 1802 plugs. The inoculated plants were maintained at high humidity conditions and suitable temperatures to facilitate infection. The conditions were maintained at 28-30 ^{0}C and 80 – 100% humidity.

Screening and evaluation of disease severity index (DSI)

Plants that were inoculated with *R. solani* 1802 were observed for a period of 10 days post inoculation (dpi). The disease severity index was recorded in triplicates. The standard DSI scale of 1-5 was used where the scale at level 1 denotes plants at high level of resistance while level 5 was assigned to highly susceptible plants that die post infection within the duration of observation (Park et al., 2008). Four

rice varieties; UKMRC2, UKMRC9, MR219 and *Oryza rufipogon* were selected for this screening activity. We screened these lines as we needed to identify the most tolerant variety. To date, no resistant variety has been developed against *R. solani* worldwide. We believe that the moderately resistant or tolerant cultivar would serve as a good donor for the isolation of the *GTP Rac* gene that may be used in functional analysis as well as in mutational studies.

Total RNA extraction

Hundred milligrams of young infected rice leaves showing low disease severity index (DSI) were harvested. Total RNA was extracted from these samples using RNeasy Plant Mini Kit (Qiagen). The protocol for extraction was as recommended by manufacturer with no modifications. The RNA was used as template for the amplification of the gene using the specifically designed primer pairs.

Amplification of Rac GTPase cDNA

The Primer3 program (http://frodo.wi.mit.edu/) was used to design specific primers with sequence from accession No: AB047313.1 as template. The cDNA product was synthesized using the above derived primer set and the RevertAid TM First Strand cDNA Synthesis Kit (Fermentas). The amplification cycle was: Denaturation at 95^0C for 2 minutes at a single cycle; Annealing at 55^0C for 12 minutes and elongation at 72^0C for 2 minutes for 35 cycles and the final elongation at the same temperature for 5 minutes. The product was then purified via QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's recommendations. The sample was then sequenced.

Characterization of Rac GTPase gene and protein

The amino acid sequence of the protein was analyzed using Conserved Domain search in http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi . The location of motifs/domains were identified through Prosite (http://web.expasy.org/prosite/) from ExPASy, (Swiss Institute of Bioinformatics) (Zdobnov and Apweiler, 2001) and Conserved Domain Architecture Retrieval Tool (http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi?cmd=rps).

For the analysis of the physico-chemical parameters of proteins, sequences were analyzed via ProtParam (http://web.expasy.org/protparam/) from ExPASy, (Swiss Institute of Bioinformatics) (Gasteiger et al., 2005). The prediction of transmembrane segments was performed using TMHMM software v2.0 (http://swissmodel.expasy.org/). The presence of signal peptides in the protein was determined via the SignalP 4.1 Program (http://www.cbs.dtu.dk/services/SignalP/)(Petersen et al., 2011). Multiple sequence alignment was performed with Multialign (http://multalin.toulouse.inra.fr/multalin/) (Notredame et al., 2000). Multiple alignment parameters were set with modification: gap opening penalty 10; gap extension penalty 1.0; delay divergent sequences 35%; protein weight matrix BLOSUM series (Henikoff and Henikoff, 1992). The phylogenetic analysis by maximum likelihood and maximum parsimony was constructed with MEGA 5.10 (http://www.megasoftware.net/index.php). The number of bootstrap replications was set as 1000. The 3D structure was predicted using I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/)(Roy et al., 2010; Roy et al., 2012). All these *in silico* analysis was conducted to obtain some level of molecular characterization of the amplified gene product.

Conclusion

Rac GTPase gene of a local rice cultivar *UKMRC9* was successfully isolated and amplified. The *Rac GTPase* UKM was approximately 1091 bp and the translated protein sequence was 307 amino acids long. The analysis conducted on 21 Rac GTPase proteins of *Oryza sativa* indicated that these sequences were highly conserved in the central region of the gene and were homologous among *Oryza* species. The phylogenetic analysis (ML and MP) of the proteins showed a monophyletic relationship between Rac GTPase UKM and Rho GTPase proteins in *Oryza sativa*. The domain analysis results further support the relationship of Rac GTPase UKM to the Rho GTPase group of proteins through the identification of actin like protein domains which indicates actin polymerization which functions in cytoskeletal arrangement and rearrangement in the eukaryote cell wall. The 3D structure of the protein exhibits the standard 6 β-sheet structure and the presence of insert region and switches which have been implicated in the ATP binding function of Rho/Rac like proteins. From this *in silico* analysis and related research, Rac GTPAses have been implicated in various biological functions in plants and therefore it is highly likely that this protein functions as a signal transducer or regulator that may regulate various biological functions in an organism.

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