Study of Putative Pathogenesis-Associated Genes of *Rhizoctonia solani* AG1-IA Causal Agent of Rice Sheath Blight

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Abstract

Rhizoctonia solani (Rs), the causal agent of rice sheath blight, is a notorious soil-borne disease prevalent in many rice-growing regions including in Indonesia. Although several studies of pathogenesis of *Rs* have been reported, there have been no reports describing the putative pathogenesis-associated genes of *Rs* AG1-IA in Indonesia. Current study stated that there were 3942 genes that were identified unique to *Rs* AG1-IA infecting rice. However, information on interaction and the pathogenicity genes of these *Rs* anastomosis groups during infection of rice is lacking. Therefore, this study is aims to do inceptive detection of putative pathogenesis-associated genes of *Rs* AG1-IA in Indonesia. Fifty *Rs* isolates were collected from thirteen districts of three rice producing provinces in Indonesia; DI Yogyakarta, Central Java, and South Sulawesi, and were identified as *Rs* AG1-IA based on the presence of 137-140 bp AG1-IA specific primers named Rs1F/Rs2R, also based on macroscopic and microscopic observation. Furthermore, the presence of the putative pathogenesis-associated genes were detected using primers Rga1, AG1_AP, AG1_PC and AG1_CFEM that were applied to selected isolates. The presence of a putative pathogenesis-associated genes was detected by the formation of amplicons at 172 bp using AG1_PC and 144 bp using primers AG1_CFEM. The associated bands showed 100% similarity to *glycosyltransferase family* 2 protein of *Rs* AG1-IA for AG1_PC primer and *clathrin adapter complex small chain domain* for AG1_CFEM primer after sequencing and analyzing by BLASTx tools. While primers Rga1 and AG1_PC didn't show a specific band. Through the present study, pathogenicity genes were found specific to *Rs* AG1-IA. AG1_PC in MAG B2 and SPG A2 isolates; and AG1_CFEM in SPG A2, KLA D1, MAG B2 and SLE B1 isolates. These two primers can be used to next step study to better understand the molecular aspect of pathogenicity in Rs AG1-IA.

Keywords : putative pathogenesis-associated genes, *Rhizoctonia solani* AG1-IA, Indonesia

Introduction

Rice (*Oryza sativa* L.) is a national strategic commodity. Most of Indonesian people use rice as a staple food source and its demand continues to increase. Therefore, efforts to increase rice production need to be carried out to meet the increasing needs and population. However, increasing rice production is still experiencing many obstacles, such as pathogen attacks that can reduce rice production both in terms of quality and quantity.

Rhizoctonia solani (Rs), the causal agent of rice sheath blight, is a notorious soil-borne disease prevalent in many rice-growing regions including in Indonesia. Rice yield loss due to sheath blight in Indonesia is 20-35% (Suharti, *et al.*, 2022). Sheath blight is well developed across all rice growing locations, both on the ecosystems of upland and lowland (Nuryanto, 2011).

Identification and classification of *Rs* is rather difficult because there are morphological similarities between strains. Likewise, identification based on anastomosis group (AG) is no longer accurate because isolates from the AG1-IA group are known to still be able to fuse with isolates from other AG1 groups (Khodayari *et al.,* 2009; Ogoshi, 1987). Although morphological and hyphal anastomosis reaction remains the main basis of identification and classification, but current molecular approaches is preferred and confirmed as reliable tools to differentiate isolates of *Rs* (Sharon *et al.*, 2008; Fang *et al*., 2013). Identification of *Rs* based on hyphal

anastomosis reactions, have weaknesses such as the reproducibility of these interactions needs experiences, is a time-consuming process and can be affected by factors including laboratory environment, nutritional conditions, and genetic stability (Carling *et al.,* 2002; Stodart *et al*., 2007). Meanwhile, techniques with molecular markers are more efficient, have high accuracy, and are more reliable than phenotypic markers, so they are widely used in the analysis of genetic diversity.

In addition to molecular identification, understanding the molecular aspects of the pathogenesis of *Rs* in its interactions with the host will enable better management of diseases caused by this pathogen (Rioux *et al.,* 2011). Current study stated that there were 3942 genes were identified unique to *Rs* AG1-IA infecting rice, but information on interaction and the pathogenicity genes of these Rs anastomosis groups during infection of rice is lacking (Ghosh *et al*, 2014; Prashantha *et al*., 2021). Rioux *et al*. (2011) designed primers for genes that are thought to be associated with the pathogenicity of *Rs* to compare between gene expression as resistance gene analog and common in fungal extracellular membrane gene etc in the AG1-IA and AG3 subgroups. Although several studies of pathogenesis of *Rs* have been reported, there have been no reports describing the putative pathogenesis-associated genes of *Rs* AG1-IA from Indonesia, so this study is aims to do inceptive detection of putative pathogenesis-associated genes of *Rs* AG1-IA in Indonesia. Furthermore, research on molecular aspects related to the pathogenicity of *Rs* is still being studied so that the information is expected to be a major contribution in the formulation of the most appropriate strategy for genetic engineering in controlling the *Rs* fungus AG1-IA, the causal agent of rice sheath blight.

Rs consist of 14 anastomosis groups which are distributed world-wide (Carling *et al.* 2002). This study use *Rs* AG1-IA because *Rs* that causes sheath blight of rice include in Indonesia is *Rs* AG1-IA. It proved by the sequencing results of 12 *Rs* isolates from Indonesia that showed a high genetic relationship with AG-1 IA tester isolates (Manasikana *et al.* 2021). It induces necrotic lesion on leaf blades and leaf sheaths of infected plant (Prashanta et al. 2021).

Material and Methods

Fungus Material. *Rs* were isolated from symptomatic plants of rice sheath blight collected from several paddy fields in thirteen districts of South Sulawesi, DI Yogyakarta and Central Java (Figure 1). All samples were marked, packaged in container and dry ice (Karen *et al.,* 2004) and brought to the laboratory for fungal isolation purpose. The rice sheath bits (4-5 mm) containing diseased and healthy sheath portions, were cut using sterilized scissors, surface sterilized in 1% hypochlorite solution for 3-5 minutes, rinsed 2-3 times in sterilized water, blotted dry and placed aseptically on potato dextrose agar (PDA) medium. Five such bits were placed on PDA in each Petri dish and incubated for two days at 28±2°C. The hyphal tips emanating from the tissue bits were cut off and transferred to PDA in Petri plates for purification and further growth of the pathogen .

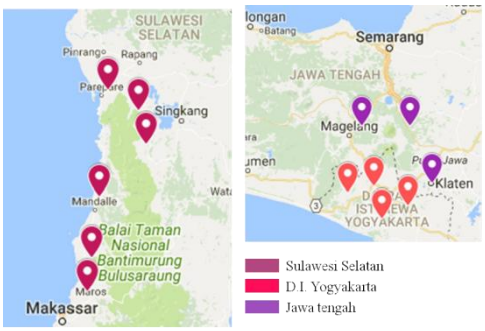

Figure 1. The Sampling Locations from 3 Provinces in Indonesia

Morphological Identification. Morphological identification of the fungus *Rs* was carried out macroscopically and microscopically. Macroscopic observations included observations of the mycelium and the presence of sclerotia, while microscopic observations included observations of the characteristics of perpendicular branches and septums observed using a microscope (Guleria *et al*., 2007; Desviani *et al.* 2018).

Fungal DNA Isolation. All isolates were purified by phenol/chloroform purification (ph/chl) (Sambrook *et al*., 1989) and subcultured from PDA to Potato Dextrose Broth (PDB) medium, incubated for 5-7 days in shaker incubator at room temperature for fungal DNA isolation using CTAB method following the procedure of Subandiyah (2003).

Molecular Identification of *Rs*. The DNA of the isolated genomes was amplified using PCR with specific primers for *Rs* spesifically subgroup AG1-IA named Rs1F (5'-GCCTTTTCTACCTTAATTTGG CAG-3') and Rs2R (5'-GTGTGTAAATTAAGTAGACAGCAAATG-3') to confirm the *Rs* AG1-IA isolates (Sayler & Yang, 2007). A master mix MyTaq™ HS Red Mix Bioline of 12.5 µl reaction was prepared with PCR components with the total volume 25 µl (master mix 12.5 µl; ddH2O or RNase free water 9.5 µl; primer forward and reverse each 1 µl; and 0.1ng/µl genomic DNA). The PCR reaction was performed by predenaturation procedure 95° C for 15 minutes, 40 PCR cycles of 95° C for 15 seconds, annealing of 52° C for 30 seconds, and extension of 72° C for 15 seconds, followed by a final extension of 72° C for 5 minutes (Sayler & Yang, 2007). The PCR product was then electrophoresed in 1.5% agarose gel at 100 Volt for 30 minutes. The presence of 137-140 bp fragments were then identified (Sayler & Yang, 2007).

Putative Pathogenesis-Associated Genes Detection. The five isolates were selected based on the results of clustering with BOXA1R and ERIC2 primers (Hamzah, 2018). Three isolates representing each cluster resulted from BOXA1R primer amplification, namely KLA D1, MRS A3 and SLE B1 isolates, while based on the results of clustering with ERIC2 primers, four representative isolates were selected, namely MRS A3, SPG A2, MAG B2 and KLA D1. KLA D1 and MRS A3 isolates were the same isolates representing both rep-PCR primers (Hamzah, 2018).

A master mix MyTaq™ HS Red Mix Bioline of 12.5 µl reaction was prepared with PCR components with the total volume 25 µl (master mix 12.5 µl; ddH2O or RNase free water 9.5 µl; primer forward and reverse each 1 µl; and 0.1ng/µl genomic DNA as template in a 0.2 ml tube. PCR reaction is conducted as the same as above.

The PCR reaction was carried out with a predenaturation of 94°C for 5 minutes, followed by a 35 cycle PCR with denaturation of 94°C for 1 minute , annealing of 55°C for 90 seconds and extension of 72°C for 2 minutes. The PCR was ended with a final elongation of 72°C for 10 minutes (Charoensopharat *et al.,* 2008). The PCR product was then electrophoresed using 1.5% agarose gel at 100 Volts for 30 minutes.

Table 1. Primers used to detect putative pathogenesis-associated genes of *Rs* AG1-IA (Rioux *et al.,* 2011*; Charoensopharat *et al.,* 2007**).

Primer	Sequence (forward- reverse)	Length (bp)
AG1_PC*	CGCTTGTTTACCGGCTGTAT GCGCTGGTATTGGTAATGCT	172
AG1_CFEM*	GCAATTCCACCTCCACTGTT TCATTCCTAAGCCTGCAACC	144
AG1_AP*	CTGCTGTTTACGGGGTTGTT TATACGTGCGTCCGAGAAG	104
Rga1**	CGGATCCAARTGGATHCAYTG YTT GGGATCCHGTRTCYGTYGCRC AYGT	514

Data Analysis. The PCR product showing a single amplicon according to the target size was sent to 1st BASE (Malaysia) for nucleotide sequencing. The nucleotide sequence data obtained were then aligned to see the homology using Multalin Tools at http://multalin.toulouse.inra.fr/ (Corpet, 1988). BLASTx analysis at https://blast.ncbi.nlm.nih.gov/Blast.cgi was performed to search for protein similarities.

Results

Morphological identification of Rs.

The results of the isolation of rice plants with symptoms of sheath blight obtained a total of 50 isolates of fungi suspected to be *Rs*. A total of 13 isolates were from South Sulawesi Province, 17 isolates from D.I Yogyakarta and 20 isolates from Central Java Province (Table 2). These provinces are three of the ten provinces that are the largest rice production centres in Indonesia (BPS, 2017). Initial identification was carried out based on morphological characters (Desvani *et al.* 2018). Observations showed that the fifty isolates had mycelium that resembled thin white threads and could change colour from cream to dark brown. The mycelium also forms sclerotia, which are small, dense aggregate structures that spread on the surface of the mycelium. The morphological characters of sample SPG A1 are shown in Figure 2. All isolates showed similar phenotypes

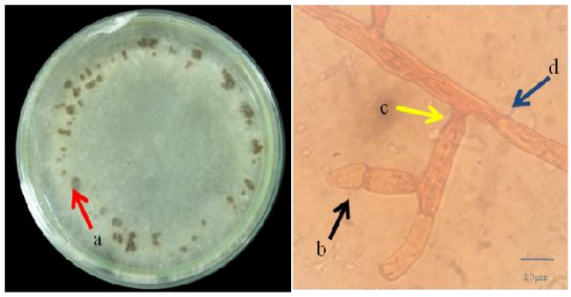

Figure 2. Colonies of *Rs* macroscopically (left) and microscopically at 100x magnification (right). Sclerotia (a), monilioid cells (b), branching hyphae (c) and septa (d).

Table 2 . Code and origin of isolates

Province	District	Isolate code
South Sulawesi	Maros	MRS A2
		MRS A3
	Pangkep	PKP A1
		PKP A2
	Barru	BRU A1
		BRU A2
		BRU A3
	Pare Pare	PAR A1
		PAR A2
	Soppeng	SPG A1
		SPG A2
	Sidrap	SDP A1
		SDP A2
D. I. Yogyakarta	Gunung Kidul	GK A1
		GK A2
		GK A3
	Sleman	SLE A1
		SLE A2
		SLE A3
		SLE B1
	Bantul	BAN A1
		BAN A2
		BAN A3
		BAN B1
	Kulonprogo	KP A3
		KP A4
		KP B1
		KP B2
		KP C1
		KP D1
Central Java	Semarang	SMG A1
		SMG A2
		SMG A4
		SMG A6
		SMG A7
		SMG A8
		SMG B1
	Magelang	MAG A1
		MAG A2
		MAG B2
		MAG C2
		MAG C3
	Klaten	KLA A1
		KLA A3
		KLA B1
		KLA B3
		KLA C1
		KLA D1
		KLA D2
		KLA D3

Molecular Identification of *Rs.*

Fifty fungal isolates were amplified using specific primers for AG1-IA subgroup, namely Rs1F/Rs2R. The results showed that the fifty isolates produced DNA bands at a size of 137-141bp (Figure 3). Based on these results, it can be said that all isolates were *Rs* subgroup AG1-IA. Sample BAN A1 is the isolate that was used as the control.

Putative Pathogenesis-Associated Genes Detection.

Five isolates were selected to be used as samples in the detection of genes suspected of being associated with pathogenicity. The five isolates were KLA D1 from Klaten, MAG B2 from Magelang, Central Java; MRS A3 from Maros, SPG A2 from Soppeng, South Sulawesi; and SLE B1 from Sleman, Yogyakarta. The five isolates were selected based on the results of clustering with BOXA1R and ERIC2 primers (Hamzah, 2018).

There are four primers used to amplify the putative pathogenesis-associated genes of *Rs* AG1-IA. These primers were primers for the genes Rga1 (Charoensopharat *et al*., 2008), AG1_AP, AG1_PC and AG1_CFEM (Rioux *et al*., 2011). The amplification results showed that the Rga1 and AG1_AP gene primers were not target specific, resulting in many DNA bands of various sizes in all the isolates used. The DNA bands produced in this study did not match the sizes stated in the literature. The target size of the Rga1 amplicon should be 514 bp while AG1_AP is 104 bp. There are no amplicons that is about the right size. The results of the primary amplification of Rga1 and AG1_AP are presented in Figure 4.

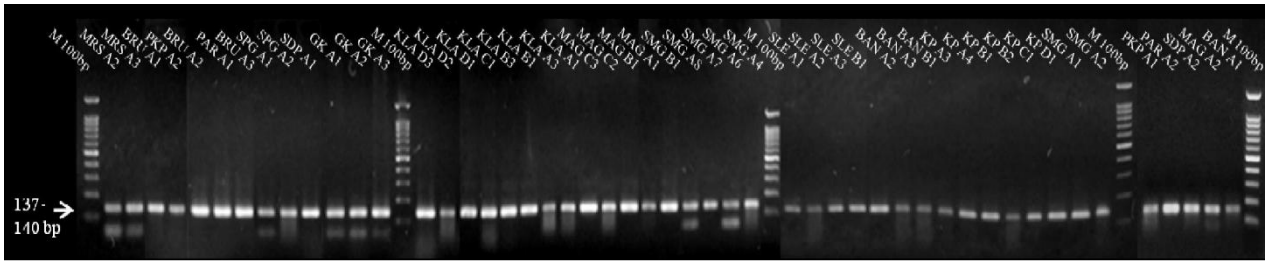

Figure 3. Visualization of amplification results with specific primer *Rs* AG1-IA

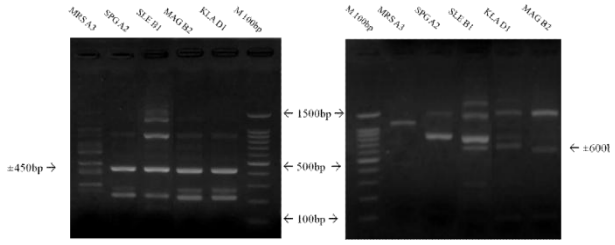

Figure 4. Visualization of amplification results with primers Rga1 (left) and AG1_AP (right) on five representatives of *Rs* isolates.

The next primers are AG1_PC and AG_CFEM primers. The target amplicon for the AG1_PC primer should be 172 bp and for AG1_CFEM it should be 144 bp. Based on the amplification results, the DNA bands formed correspond to the target sizes mentioned in the literature. Using AG1_PC primer, the isolates that present single amplicon in correct size is MAG B2 and SPG A2 isolates. While SLE B1, KLA D1, and MRS A3 showed other amplicons that is not accordance with the reference (>500bp) that may be caused by contamination (Figure 5). Single amplicon of MAG B2 and SPG A2 isolates were sent to 1st BASE (Malaysia) for nucleotide sequencing.

Similar things were also observed using AG1_CFEM primers, samples MAG B2, KLA D1, SLE B1 and SPG A2 showed single amplicon in right size is about 144 bp, while MRS A3 isolates showed unclear band and other amplicons that is not accordance with the reference (Figure 5).

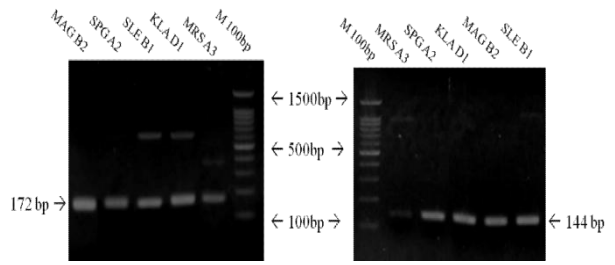

Figure 5. Visualization of amplification results with primers AG1_PC (left) and AG1_CFEM (right) on five representatives of *Rs* isolates.

Sequencing was performed on DNA fragments from isolates with single amplicons, namely isolates MAG B2 and SPG A2 with primers AG1_PC and isolates MAG B2, KLA D1, SPG A2, and SLE B1 with primers AG1_CFEM. The DNA sequences were aligned using the site http://multalin.toulouse.inra.fr/ (Corpet, 1988), to see differences in nucleotide sequences between samples. The alignment results showed that there were several different nucleotide bases in the two samples amplified using the AG1_PC primer (Figure 6) and in the four samples amplified using the AG1_CFEM primer (Figure 7). This shows that the genetic variability of *Rs* is relatively high because it has differences in the level of nucleotide bases.

Next, an analysis was carried out using the BLASTx tool on NCBI, namely the alignment of the nucleotide sequenced results with the protein data in the GeneBank database. The analysis showed that with primer AG1_PC, the sequences in the isolate MAG B2 had a similarity of 100% with the *glycosyltransferase family 2 protein* of *Rhizoctonia solani* AG1-IA, while the isolate SPG A2 had a 87% similarity with the E3-ubiquitin protein ligase. The results of BLASTx analysis on four isolates amplified using AG1_CFEM primers showed that all four had 100% similarity with the *clathrin adapter complex small chain domain*.

Based on the amplification results, it can be said that of the four primers used for the detection of putative pathogenesis-associated genes of *Rs* AG1-IA, were primers AG1_PC and AG1_CFEM. These primers amplified the gene sequences encode proteins that are putative to be involved in the expression of pathogenicity in the isolates used in this study, namely *Glycosyltransferase, E3 ubiquitin protein ligase* and *chlatrin protein*.

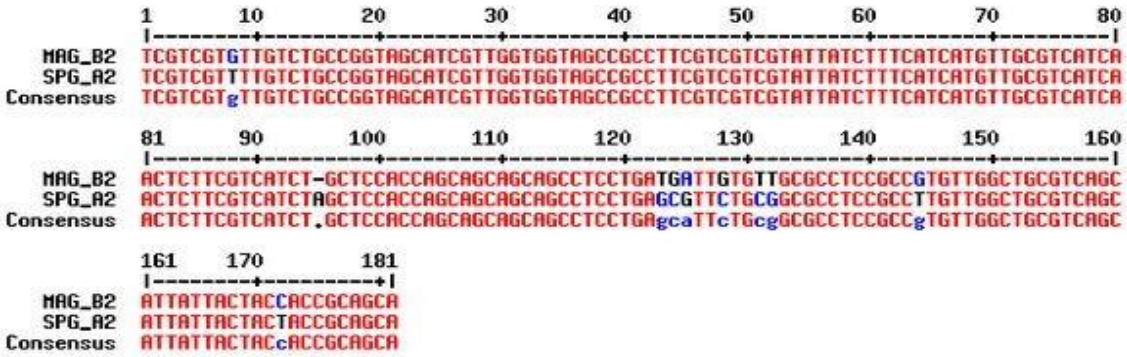

Figure 6. Nucleotide sequence alignment of amplicons amplified from isolates of MAG B2 and SPG A2 using AG1_PC primers.

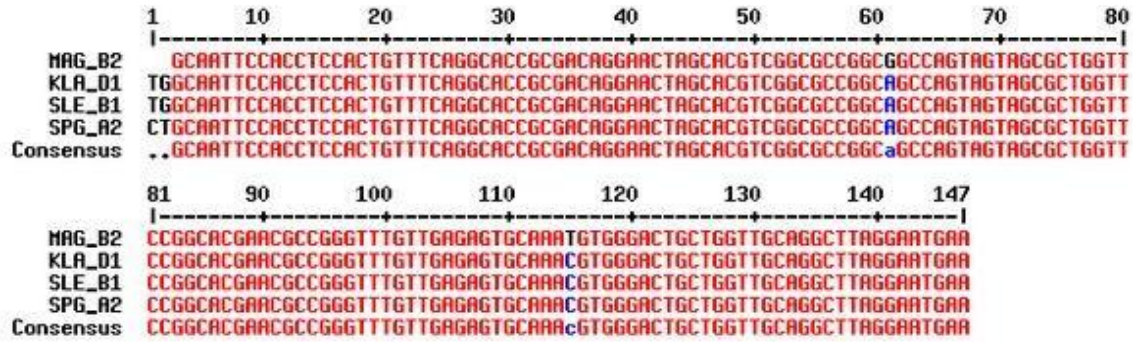

Figure 7. Multiple sequence alignment of amplicons amplified from isolates MAG B2, KLA D1, SLE B1 and SPG A2 using AG1_CFEM primers

Discussions

A total of 50 isolates *Rs* were isolated from 3 provinces in Indonesia. Figure 2 shows that macroscopically, the isolates were consistent with the general description of *Rs* characterized by the presences of hyphae and sclerotia. Microscopic observations of the fifty isolates supported the macroscopic observation. Hyphae with perpendicular branches, partitions, the presence of monilioid cells, the absence of sexual reproduction structures and the absence of clamp connections indicated that all isolates were *Rs* (Ogoshi, 1987). According to Soenartiningsih *et al*. (2015), the hyphae diameter of the fungus *Rs* depends on the isolate and the type of medium used. The hyphae of *Rs* grown on PDA medium has a diameter of 4-6 µm, while those grown on Hopkins synthetic medium can reach 6-13 µm. Hyphae of *Rs* can form mycelium consisting of monilioid cells that play a role in the formation of sclerotia (Ou, 1985). Sclerotia play a role in the survival of *Rs* in the field, it serve as the primary source of infection for rice sheath blight (Feng *et al*., 2017). Based on the observation above, it can be concluded that the 50 isolates were Rs.

Based on the molecular identification, the fifty isolates were identified as *Rs* AG1-IA. According to Jayaprakashvel & Mathivanan (2012), *Rs* which causes sheath blight in rice is classified into the subgroup AG1-IA so that for identification, the specific primer *Rs* AG1-IA is used. Sayler & Yang (2007) designed primers Rs1F and Rs2R specific to *Rs* AG1-IA. The primer was designed based on the differences in the 18-28S ribosomal DNA (r)DNA sequence of the internal transcribed spacer (ITS) region of *Rs* AG1-IA from those of *Rhizoctonia-oryzae, Rhizoctonia oryzae sativa* and *Rhizoctonia solani* belonging to different subgroups. PCR amplification using primers specific to Rs AG1-IA subgroup resulted in amplicon at approximately 137-141 bp for *Rs* from AG1-AI subgroups, with no amplification obtained from *Rs* from other subgroups (Sayler & Yang, 2007).

Furthermore, based on research on the variability of *Rs* (Hamzah, 2018), five representative isolates were used for the detection of genes suspected of being associated with pathogenicity using primers for the genes Rga1, AG1_AP, AG1_PC and AG1_CFEM (Charoensopharat *et al*., 2008; Rioux *et al*., 2011). Rga1 is a gene encoding GTP-Binding protein (G-protein), which is one of the important proteins in the transduction pathway

that regulates variability of function in eukaryotic organisms (Aukkanit *et al*., 2004). Mutations in the Rga1 affect the pathogenicity of the fungus *Rs* (Charoensopharat *et al*., 2008). Therefore, this gene is thought to be one of the genes associated with pathogenicity in the fungus *Rs*.

AG1_AP primers were designed by Rioux *et al*. (2011). The primer amplifies the homologous gene AVR_pita which acts as an effector/homologous avirulent gene in *Magnaporthe oryzae*. These genes also play a role in the supply of pathogenic nutrients and in the process of host invasion (Rioux *et al*., 2011).

In contrast to the amplification results using primers Rga1 and AG_AP, primers AG_PC and AG_CFEM showed the presence of a single band in some isolates: MAG B2 and SPG A2 for AG1_PC primers, and SPG A2, KLA D1, MAG B2 and SLE B1 for AG1_CFEM primers. According to Rioux *et al*. (2011), the AG1_PC primer is a primer that amplifies the gene encoding the pyruvate carboxylase enzyme that plays a role in metabolic processes and gluconeogenesis. The enzyme is thought to play an important role in the necrotic phase of the pathogenicity of *Rs*, while the AG1_CFEM primer is the encoding for the common in fungal extracellular membrane (CFEM). Zhang *et al*. (2015) stated that the CFEM gene is only present in certain fungi such as *Colletotricum graminicola* (Gong *et al,* 2020), *Magnaporthe Oryzae* (Kou *et al*, 2017) and *Arthrobotrys oligospora* (Zhang *et al.* 2015). The function of this gene is still not clearly understood but it is suspected as a mediator in the interaction process between the host and the pathogen in the pathogenesis process. CFEM plays a role in the contact process with the host and the formation of appressorium (Rioux *et al*., 2011). Appresorium is hyphae complex, that swells at the tip, which is useful for attaching and penetrating the host (Taheri & Tarighi, 2011).

According to Zheng *et al*. (2013) *glycosyltransferase family 2 proteins Rhizoctonia solani* AG1-IA and *E3-ubiquitin protein ligase* are genes that are putative to be associated with pathogenicity in *Rs*, namely as effector candidates that cause necrotic rice in rice.

The results of BLASTx analysis on four isolates amplified using AG1_CFEM primers showed that all four had 100% similarity with the clathrin adapter complex small chain domain. Clathrin is a protein that plays a role in the formation of layers on vesicles for the transport of molecules between cells. Clathrin must bind to the adapter to coat the vesicle in both endocytosis and exocytosis. The endocytosis pathway can be used by pathogens to enter cells during infection (The European Bioinformatics Institute, 2007). Amplified fragment as predicted for two primers (AG1_PC and AG1_CFEM) indicate that the primers encode the gene sequence specifically in Rs AG1_IA certain isolate, while two other primers were not. Regarding the unspesific bands presence from amplification with all primers, its possible that the primers complementary to repetitive DNA may produce many non-specific bands in single-primer amplification and compromise the performance of unique PCRs.

The pathogenic mechanism of *Rs* is not simple. This mechanism not only uses several lytic and degradative enzymes for infection but is thought to involve other molecules of the glycoside hydrolase (GH) enzyme group, secondary metabolites and various effectors to suppress host defences in the early stages of infection. In addition, no gene has been identified that is fully responsible for the pathogenicity process of *Rs* (Rioux *et al*., 2011; Taheri & Tarighi, 2011).

It is expected that knowing the pathogenesis-associated genes of *Rs* AG1-IA can be useful as a new target for development of novel high-effiency fungicides for controlling of *Rs* AG1-IA in rice as the research that conducted by Wang *et al*., (2022) that found the gene functional analysis of trehalose metabolism that related to sclerotial development. To conduct the long-term management of *Rs,* besides the study about detection of pathogenicity genes, we can also study about the present of resistance genes in rice. It can be useful in breeding rice varieties resistance, as the study conducted by Fatimah *et al.*(2018) which detect the resistance genes to BLB disease in local varieties of rice named *Xa7*.

Research about molecular detection, especially about the genes, should be continued by looking at the expression of the genes using reverse-transcription-quantitative PCR (RT-qPCR) method as done by Soheili-Moghaddam *et al.* (2022), that identify the novel associations of candidate genes with resistance to *Rhizoctonia solani* AG-3PT in *Solanum tuberosum* stem canker. To obtain the better study, it can be added with a method in the form of pathogenicity test in the rice. The gene detection that has been carried out is expected to

be the initial step in understanding the molecular aspects and mechanisms of the pathogenesis of *Rs* better.

Conclusively, the inceptive detection of putative pathogenesis-associated genes of *Rs* AG1-IA in Indonesia has been carried out. Study about AG1_PC and AG1_CFEM genes can be the initial research to better understanding about pathogenesis-associated genes of *Rs* AG1-IA in Indonesia as a way to manage the disease better.

There are differences between the accessions tested, based on the alignment of multiple amplicon sequences amplified from isolates MAG B2, KLA D1, SLE B1 and SPG A2 using the primer AG1_CFEM, it can be seen that KLA D1 is similar to SLE B1. This may indicate that geographic distribution matters. KLA D1 from Klaten is close to SLE B1 from Sleman.

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