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Original research article

Expression Study of Banana Pathogenic Resistance Genes

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ABSTRACT

Banana is one of the world's most important trade commodities. However, infection of banana pathogenic fungi (*Fusarium oxysporum* race 4) is one of the major causes of decreasing production in Indonesia. Genetic engineering has become an alternative way to control this problem by isolating genes that involved in plant defense mechanism against pathogens. Two of the important genes are *API5* and *ChiI1*, each gene encodes apoptosis inhibitory protein and chitinase enzymes. The purpose of this study was to study the expression of *API5* and *ChiI1* genes as candidate pathogenic resistance genes. The amplified fragments were then cloned, sequenced, and confirmed with in silico studies. Based on sequence analysis, it is showed that partial *API5* gene has putative transactivation domain and *ChiI1* has 9 chitinase family GH19 protein motifs. Data obtained from this study will contribute in banana genetic improvement.

1. Introduction

Indonesia as one of the mega biodiversity country has been known as a fruit producer for local or global consumption. Indonesia is one of the main banana producer countries in the world. However, the export of fresh fruit was only about 10% of total local production (Purwadaria 2006). This is because of several problems that occur at preharvest and postharvest stages. One of the preharvest problems is susceptibility to pathogen attack, such as bacterial, viral, and fungal infections. One of major issues in banana disease is banana wilt caused by pathogenic fungi, *Fusarium oxysporum* f.sp. *cubense* (*Foc*) (Dimyati *et al.* 2001). As soilborne fungi, the spore of *Fusarium* stays on the soil and is hard to eradicate (Walduck and Daly 2006). Therefore, this disease has led to a loss harvest of 20.000 tons in Lampung province in 1993–1994 (Dimyati *et al.* 2001).

Foc as soilborne fungi infects plants through the roots and then colonizes the vascular tissues in the rhizome and pseudostem and finally causing plant wilt that can cause plant death (Beckman 1987, 1990). Wilting symptoms on infected plants usually occur in the same time with necrosis and rotting in the root, rhizome, and the vessels of pseudostem (Pérez-vicente 2004). Banana disease treatment so far still uses conventional methods such as conventional breeding, pesticide usage, optimization of bananas planting technical process, and utilization of biocontrol agents. However, most of the commercial banana possessing triploids (AAA) are sterile, thus hybridization is less effective (Heslop-Harrison 2011). Therefore, genetic engineering has become the most promising method to be an alternative solution to solve these diseases (Gurr and Rushton 2005). Transgenic methods in developing crops such as tobacco, potato, and cucumber have chosen to express pathogen resistance-related genes (Reimann-Philipp and Beachy 1993).

Previous studies have shown that transgene could result in *Foc* resistance in banana plants. For instance, antiapoptosis genes that are isolated from animal cells can be used as resistance gene to *Foc* infection in tobacco plants, tomatoes, and bananas (Li *et al.* 2010; Paul *et al.* 2011). As an alternative of antiapoptosis genes from animal cells, we found that plant antiapoptosis inhibitor 5 (*API5*) genes were also available at the genome database of *Arabidopsis thaliana*. In addition, class I chitinase gene (*ChiI1*) from *Musa* AB group (FJ858155.2) was also available at GenBank. This gene encodes chitinase, which is an enzyme that has a role in degrading chitin component on fungi cell wall. Therefore, this research was focused to study *A. thaliana API5* and local banana (pisang ambon lumut, *Musa acuminata* AAA group) *ChiI1 gene* expressions before isolation of full-length genes. The results are expected to be a reference for conducting overexpression of both genes to produce

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transgenic banana plants that are resistant to *Foc* attack. The resistance of bananas to *Fusarium* pathogens is expected to improve the quality and quantity of banana production in Indonesia. This can provide benefits for banana farmers in Indonesia, increasing export and support agroindustry by reducing production losses caused by pathogen attack.

2. Materials and Methods

2.1. Gene expression study

Gene expression study was done by reverse transcription polymerase chain reaction to amplify *ChiI1* from pisang ambon lumut *(M. acuminata* AAA group*)* leaf complementary DNA (cDNA) using the method of Handayani and Dwivany (2014) and Dwivany *et al.* (2015). Primers were designed based on sequences of class I chitinase gene (*ChiI1*) *Musa* AB group (FJ858155.2). The primers were 5′(GACTGCTAAGGAGGATGAAG)3' as forward primer and 5′(GGCCATGTACTAGTTCAGC)3′ as reverse primer. Meanwhile, to study expression of *API5*, root cDNA of *A. thaliana* (12 leaves stage plants) was used as template. Primers were designed based on sequences of *A. thaliana API5* (NM_128955.4). The primers were 5′(AATTTCATCAGAGATAAGGTGA)3' as forward primer and 5′(ACTTGTTATTACCAGTGACC)3' as reverse primer.

2.2. Gene characterization

The cDNA fragments were then sequenced and analyzed through in silico studies. Web sites and software used are Softberry© (Softberry, Inc., http://www.softberry.com), analysis of protein localization at sites CELLO (http://www.cello.life.nctu.edu.tw/cgi/main.cgi), analysis of local alignment (BLAST) from the GenBank Web site (http://www.ncbi.nlm.nih.gov), and multiple sequence alignment with ClustalW (http://www.srs.ebi.ac.uk). Gene annotations were also conducted with CLC Genomics Workbench v3.6.5© software (QIAGEN Bioinformatics).

3. Results

3.1. Gene expression study

The ~1178 bp *API5* was amplified from root tissue cDNA (Figures 1A and 1C). This result shows that *API5* was expressed in the root of *A. thaliana* plant. Meanwhile, ~1053 bp fragment was amplified using *ChiI1* gene-specific primers from leaf cDNA (Figures 1B and 1D). This result showed that the genes were expressed in banana leaf tissue.

3.2. Gene characterization

Motifs from each sequence were characterized with in silico studies. Figure 2A shows that putative API5 amino acid sequences have the same motifs with API5 from another species such as *Oryza sativa* and *Homo sapiens*. In the figure, one represent as LXXLL motif, two represent as transactivation domain, whereas three represent as nuclear localization sequence. From the alignment, putative API5 shares partial amino acid sequences characterized as transactivation domain.

As for the chitinase, it has been found that several motifs are based on the multiple sequence alignment result (Figure 2B). Motifs 1, 2, 3, 5, 12, and 13 are chitinase protein motifs found only in plants, whereas others may be found in another species, such as arthropods, purple bacteria, actinobacteria, and nematodes (Udaya Prakash *et al.* 2010).

4. Discussion

To identify putative genes, both cDNA fragments were cloned and sequenced, and the sequencing results were then analyzed with BLAST. The sequences were aligned to both nucleotide and protein database in GenBank. Alignment results showed that putative genes of *API5* share high homology with *API5* gene and protein from *A. thaliana*. Based on nucleotide alignment (BLASTn), putative *API5* gene shares 90% homology with *A. thaliana* apoptosis inhibitory protein 5 (*API5*) (NM_128955.4). In addition, putative *API5* gene sequence was also compared with protein database in GenBank using BLASTx alignment. Predicted amino acid sequence of putative *API5* gene shares 96% homology to API5 protein isolated from *A. thaliana*. Because of this sequence similarity, putative *API5* that has been successfully isolated is considered as one of the *API5* gene (NP_565777.1). *API5* genes were also compared for its amino acid motifs and protein domain using characteristics compared from other known *API5* gene family, specifically from *Oryza sativa* and *Homo sapiens* (Li *et al.* 2011). From Figure 2A, putative API5, both *Os*API5 and *Hs*API5 have conservative motifs, such as LxxLL motif, transactivation domain, nuclear localization sequence, and a

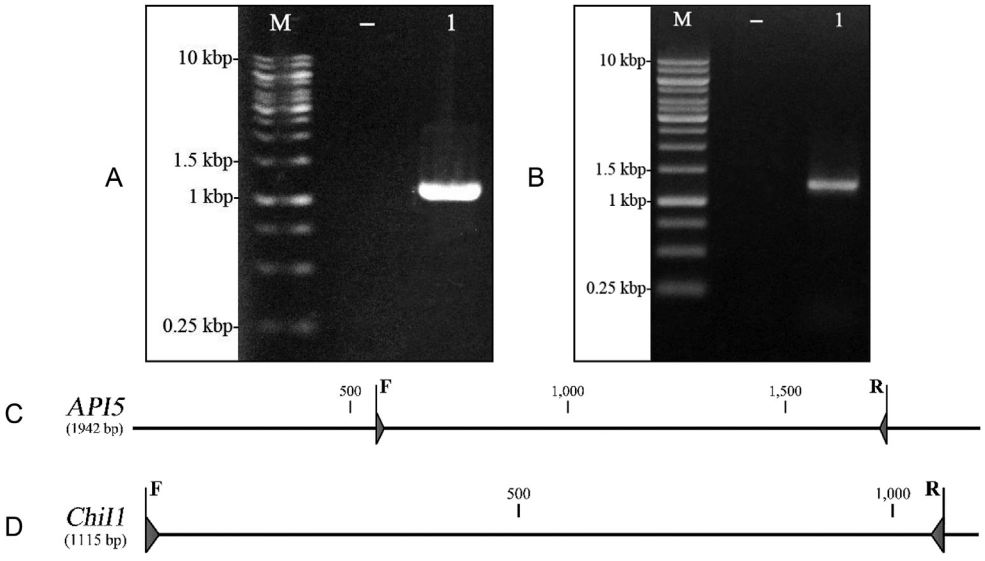

Figure 1. PCR result of *API5* gene amplification using touchdown (A) PCR method and (B) *ChiI1* gene from cDNA pisang ambon lumut. (C) and (D) show primers position for each gene amplification. PCR, polymerase gene reaction.

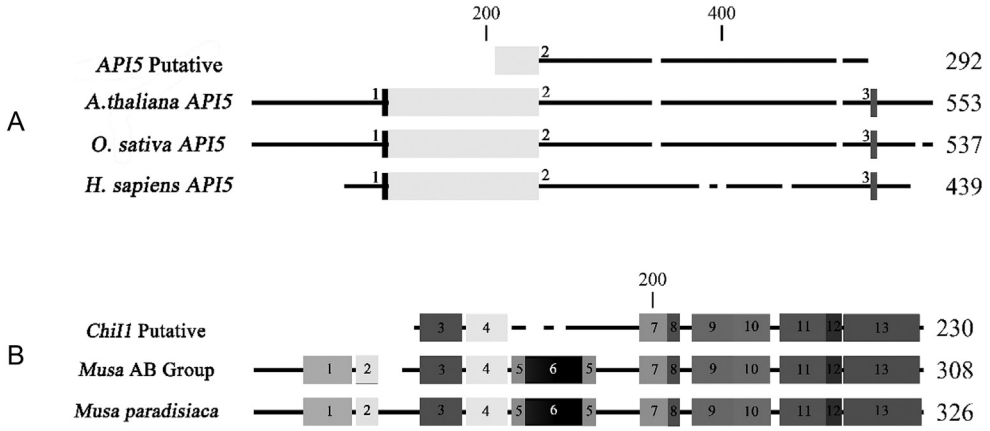

Figure 2. (A) Multiple sequence alignment between putative *At*API5 and another API5 protein from *Oryza sativa* (*Os*API5) and *Homo sapiens* (*Hs*API5). (B) GH19 chitinase family protein motifs found on reference gene sequence (*Musa* AB group, *Musa paradisiaca*) and isolated gene fragment from pisang ambon lumut. These motifs are (1) [RS]QAGGALCP[NG]GLCCS [QKE][FY]GWCG[SN]; (2) [CK][GQ]PGCQSQC[GTS][GP]; (3) [IL]IS[RS]S[LQ]F [DN][QD]ML[KL]HRND[AG]ACP[AG]; (4) FYTY[DN][AG][FL][IV][AT]AAK [SA]FP[GA]F[GA][TN]TG; (5) E[IV]AAF[LF][GA]QTSHETTGGW[AP][TS]APD GP[YF][AS]WGYCF; (6) DA[ITV]CK[RK][ES][LAI]A[AT]F[LF]A[NQH][VF] [SA][HQ]E[TS]GG[LH]x[YA][VI]VExN; (7) [FHY]-G-R-G-[AP]-x-Q-[IL]-[ST]-[FHYW]-[HN]-[FY]-NY; (8) Y[YF]GRGPIQ[LI][ST][WY]N[YF]NYG[AP][AC] GRA; (9) NNPDLVA[TN]D[AP][VT][IV][SA][FW]KTA[LI]WFW; (10) MT[PA]Q [SP]PKPSCHDVITG [RQ]W; (11) DSAAGR[LVG][PA]G[YF]GV[IT][TI]NIINGG [LI]EC; (12) G[RK]GQDSRV; and (13) [DN]RIGF[YF][KQ]RYCD[IL][LF]GV [GS][YP]G[DN]NLDCYNQR[PS]F.

leucine zipper (Plevin *et al.* 2005; Li *et al.* 2011). *Hs*API5 also predicted to have such motifs, but it was found that there are differences from its amino acid residues. This comparison of motifs shows that putative gene that has been isolated from cDNA of *A. thaliana*^{WT} is suitable as one of the members of *API5* gene because it has transactivation domain, a characteristic of anti-apoptosis gene.

The 1053 bp *ChiI1* cDNA sequence was also compared with BLASTn method. BLASTn results showed that the isolated base sequence has a sequence similarity with class I chitinase genes in *Musa* AB group (94%) and partial sequence of *M. acuminata* endo-chitinase (98%). Chitinase is found in a wide range of organisms: bacteria, viruses, animals, and higher plants. Chitinase class I has a chitin-binding domain that is rich with amino acid cysteine at the N terminus and a catalytic region at C terminus, and all these are connected through the linker that has a short peptide of 10–20 amino acid residues. Chitinase class II only has a catalytic region homologous to chitinase class I. Chitinase class IV has homology with class I. Chitinase class III and V show no similarities with class I, II, and IV but has obvious sequence similarity with chitinase sequences belonging to bacteria and fungi (Funkhouser and Aronson, 2007; Seidl 2008). Henrissat and Bairoch (1993) have proposed a classification system for glycosyl hydrolase enzymes. Based on this classification, the enzyme chitinase classes I, II, and IV were classified as family 19, whereas chitinases class III, IIIb, and V were grouped into family 18.

Predicted amino acid sequence of the isolated gene fragment was performed using BioEdit software version 7.1.3.0 (Ibis Biosciences, http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and amino acid sequence prediction program on http://www.softberry.com site. This amino acid sequence then aligned with the amino acid sequence of *Musa* AB group that was used as a reference in designing primers for gene amplification and *Musa paradisiaca*. Both of them have full-length chitinase gene sequences (http://ncbi.nlm.nih.gov). This alignment was done to find and compare conserved protein motif of GH19 chitinase family on the isolated gene fragment and sequence references (Figure 2). Based on the protein motif analysis, there are 13 protein motifs of GH19 chitinase family present in the reference gene sequence, *Musa* AB group, and *M. paradisiacal*, whereas there are only nine family GH19 chitinase motifs found in the isolated chitinase gene fragment from pisang ambon lumut. The six GH19 chitinase motifs are typical of plant chitinase, whereas three other motifs are highly conserved through GH19 family chitinase. These motifs support that the gene fragment isolated from pisang ambon lumut is a strong candidate of class I chitinase gene family GH19.

Based on the expression study, *API5* gene was expressed in *A. thaliana* root and has putative transactivation domain. On the other hand, *ChiI1* gene was expressed in pisang ambon lumut leaf (*M. acuminata* AAA group) and has chitinase family GH19 protein motifs. Further gene isolation and characterization need to be done to obtain full-length resistance genes and their protein functions. Therefore, it can be used as a promising candidate of resistance genes to produce pathogen-resistant plants. For further research, overexpression of these genes need to be done to determine if it confers to resistance to *Foc*.

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Conflicts of Interest

The authors declare no conflict of interest.

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