Molecular analysis of gene from ginger and *Zingiber zerumbet* encoding resistance to bacterial wilt

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Abstract. The bottleneck in ginger cultivation is bacterial wilt which causes crop damage of more than 70%. Since conventional cross-breeding in ginger is difficult, genetic engineering has allowed new ginger variety resistance to bacterial wilt development. Thus, a homologous sequence of resistance genes (RRS1-R) could be designed and constructed, then transformed to generate new ginger variety tolerance to *R. solanacearum*. The more resistant genotypes (red ginger and wild ginger/shampoo ginger) were subjected to gene isolation in this research. The red and wild ginger species were first inoculated with a suspension of *R. solanacearum* before gene isolation. Then, the generated primer was used to isolate homologous sequences of RRS1-R gene candidates from both species. Cloning and sequence results showed that induced red and wild ginger tissues to *R. solanacearum* were expressed on both species. However, the RRS1-R homologous gene was not detected. Furthermore, the full-length DNA gene cloned from the red and wild ginger species was homologous to the Kafirin gene group. These results indicated that different genes might have been involved in encoding resistance to bacterial wilt in ginger.

Keywords: cloning, gene characterization, gene isolation, RT-PCR, sequencing

1. Introduction

Ginger is a potentially herbaceous crop that takes advantage of uses in medicinal properties for various diseases. However, one of the major constraints in ginger cultivation is bacterial wilts caused by *Ralstonia solanacearum*. So far, efforts in the development of ginger variety resistance to bacterial infections have not worked significantly. The reasons are two-fold. First, the improvement of ginger varieties for resistance to bacterial wilt disease is very complicated. It is strongly influenced by various factors, *e.g.*, the source of resistance genes, a correlation between resistance traits and other agronomic characters, differences in pathogenicity between strains, interaction mechanisms between pathogens and plants, and breeding methods used. Second, the genetic diversity of ginger is relatively low because it is a non-native plant and propagated vegetatively. Thus, the opportunity to obtain a source of genes for resistance to bacterial wilt disease or other essential traits is getting smaller. Meanwhile, genetic engineering technology provides a new vision for plant breeders to obtain a new gene. The sequential DNA of a new gene, which is transferred into the genome of a plant to form transgenic plants, can be derived from other plant species, bacteria, or viruses. More specifically, the discovery of genes encoding resistance to *R. solanacearum* (RSS1-R) in *Arabidopsis thaliana* [1, 2, 3], combined with a bioinformatics approach and molecular biology techniques, made it possible to isolate a gene from...
tolerant ginger plants. Later, the homology gene could be constructed and transformed to form new
ginger varieties resistant to bacterial wilt. In addition, it was reported that based on the sequence analysis,
several domains of the RRS1-R gene had an essential function in the resistance mechanism, possibly
mediated by signal transduction of salicylic acid [4].

Results of germplasm evaluation showed that red ginger and its wild-related species (Zingiber spp.)
were more tolerant to bacterial wilt than white ginger [5, 6]. Therefore, the opportunity to assemble
resistant varieties to bacterial wilt using genetic engineering is open. However, unfortunately, up-to-date
data on the resistance of ginger to bacterial wilt disease are yet available. Therefore, this study was
aimed to observe the homology of resistance genes to bacterial wilt contained in the ginger genome and
its wild relatives. Results obtained will serve as the basic information in tracing the source of resistance
genes to develop a tolerant variety to bacterial wilt.

2. Material and methods
2.1. Plant materials and chemicals used
The plant materials used were big-white ginger var. Cimanggu 1 (Zingiber officinale var. officinale),
red ginger var. Jahira 2 (Z. officinale var. rubrum), and shampoo ginger (Z. zerumbet var. zerumbet).
Red ginger and shampoo ginger were used as genetic sources for gene isolation because, based on the
results of previous studies, both genotypes were more tolerant to bacterial wilt disease than big-white
ginger. Big-white ginger, red ginger, and shampoo ginger used in this study were obtained from the
RISMC germplasm collection. The three genotypes to be tested were planted in polybags of 20 plants
each. Then, inoculated with a suspension of R. solanacearum after the plants grew normally with 5-6
leaves (about three months old).

2.2. Inoculation procedures
First, a suspension of the bacterial wilt disease pathogen R. solanacearum with a concentration of 10⁷
cfu/ml was prepared. Then, inoculation was done by sprinkling 250 ml of the suspension on polybag
growing media containing 500 g of soil, planted with three-month-old ginger plants.

2.3. Total RNA isolation and primer design
Total RNA was isolated using a method developed by Chaidamsari [7], whereas the primer was designed
using the primer3 program available on the website http://frodo.mit.edu/.

2.4. First-strand DNA synthesis
The first strand of cDNA synthesized was performed using the SuperScriptTM II Reverse Transcriptase
(Invitrogen) kit. The reaction volume is 20 L for every one ng to 5 g of total RNA. The heating and
incubation process was carried out using a PCR (Biometra T-personal) machine. The synthesis was
carried out in sterile micro-tubes with the composition of 9 L ddH2O, 1 L 10 mM dNTP mix, 1 g ginger
leaf total RNA, and 1 10 p mol/μL oligo (dT)23 (Ambion). The mixture was heated at 65 °C for 5
minutes and immediately put into an ice bath, then 4 L of first-strand buffer 5X and 2 L 0.1 M DTT
were added. Mixed immediately and incubated at 42 °C for 2 min. Then 1 L (200 units) of
SuperscriptTM II Reverse Transcriptase enzyme was added and mixed by pipetting up and down. After
incubation at 42 °C for 50 minutes, the reaction was inactivated by heating at 70 °C for 15 minutes, then
1 L (2 units) of RNase H was added and incubated at 37 °C for 20 minutes. The first-stranded cDNA
formed was then used as a template for amplifying the RRS1-R gene fragment, using the RRS1-R-
specific primer.

2.5. Reverse transcriptase-PCR
The template used in the reaction was total ginger cDNA first strand, and the procedure used was a
modified Superscript TM II RT (Invitrogen). RT-PCR was carried out with a PCR mix volume of 25 μL
consisting of: 2.5 μL 10x complete buffer, 1 μL dNTPs, 1 μL primer F, 1 μL primer R, 0.5 μL Taq
polymerase, 1 μL template, 18 μL molecular water. RT-PCR amplification was performed using the T-
personal Biometra PCR machine with the following program: initial denaturation at 94 °C for 5 minutes; denaturation at 94 °C for 45 seconds; annealing at 50 °C for 45 seconds; extension 72 °C for 5 minutes. The amplification results were verified on 1% agarose gel.

2.6. Extraction and purification of amplicon
The procedure used was the Axyprep DNA gel extraction kit (Axygen). The DNA bands of the expected size were then extracted from the gel. The extraction results were verified as much as 2 L on 1% agarose gel, and if positive, then ligated to the cloning vector.

2.7. Cloning DNA fragments of the RRS1-R gene with pGEM-T easy
The amplification results were purified using Axyprep DNA gel extraction kit (Axygen) and inserted into the pGEM-T Easy (Promega) vector using T4 DNA ligase for one hour, 37 ºC. The vector was then transformed into competent-E. coli XL-1 Blue. Competent cell preparations were carried out according to the procedure of Inoue et al. [8]. The ligation mixture added to 200 µL of competent cell suspension was 20 µL. Cells were then spread on LB (Lysogeny Broth/Luria Bertani) agar solid media containing 100 mg/L ampicillin, 40 mg/L X-Gal, and 0.1 mM IPTG (LA) antibiotics then incubated overnight at 37ºC.

2.8. PCR colony
There were two types of colonies formed on the petri dish. The white colonies indicated that cells contain plasmids that have been successfully inserted. The blue colonies showed that cells contained plasmids that were not successfully inserted. Each white colony formed is numbered and marked. The white colonies were then taken with a toothpick for colony PCR, slightly transferred to LB agar medium in a petri dish. The remainder was put into LB medium containing liquid ampicillin to be re-cultured overnight at 37ºC, 150 rpm.

2.9. Miniprep DNA plasmids
High Pure Plasmid Isolation Kit (Roche) was used for the isolation of plasmid DNA. The isolation was carried out on the known PCR colonies to contain the desired plasmid inserted fragment. The colonies were cultured in LB + liquid ampicillin overnight at 37ºC and 150 rpm with agitation. Liquid cultures were labeled according to the respective colony labels. The plasmid DNA obtained was verified on 1% agarose gel to see its purity.

2.10. DNA sequencing
Sequencing was carried out at the Eijkman-Jakarta institute. First, DNA was sequenced using universal primers M13-F and M13-R. The obtained DNA sequences were then analyzed by a bioinformatics program available at (www.ncbi.nlm.nih.gov).

2.11. Isolation of the full-length gene RRS1-R
The full-length gene RRS1-R was obtained from 5’RACE and 3’RACE cloned and then sequenced. Once the full length of the RRS1-R gene has been obtained, it is ligated to the expression vector under the 35SCaMV promoter.

2.12. Characterization of the RRS1-R gene in various plant tissues
The plant DNA fragments that have been identified and sequenced are then designed for primers for RT-PCR. The tissue that will be characterized to determine the presence of the RRS1-R gene was isolated for RNA. The tissues used include roots, leaves, rhizomes. Isolation was carried out according to the procedure described previously. Furthermore, the results of the isolation were re-analyzed using PCR.
3. Results and discussion

RT-PCR was first carried out on the total RNA of the samples without inoculation (data not shown). It was observed that the resistance gene to bacterial wilt was not expressed in all samples tissues. This showed that the nature of resistance to *R. solanacearum* in red ginger and shampoo ginger might be classified as a resistance mechanism that is not expressed until a pathogen attack occurs [9]. Therefore, the next step was to isolate DNA from the induced plants after the plants were inoculated with a suspension of the pathogen *R. solanacearum*, then amplified.

The visual response of plants genotypes inoculated with a suspension of *R. solanacearum* is shown in Figure 1. Big-white ginger showed severe wilting symptoms (>70% wilting). In contrast, the red ginger wilting symptoms were lower (<10%), and shampoo ginger showed no wilting symptoms (0%) at ten days after the plants were inoculated (Figure 1). Thus, symptoms of wilt in ginger plants after wilt disease infestation appeared seven days after injection. In other varieties of ginger plant inoculated with a mycelial suspension of *Fusarium oxysporum f.sp. zingiberi*, the yellows and vascular wilting, root rot, and dumping-off symptoms were appeared within three days [10]. These differences might be due to different causal agents and modes of action for the specified pathogen in ginger. Therefore, DNA samples from roots, stems, and leaves were isolated on the 10th day after injection for expression testing. Gene expression was then checked by compiling a new primer based on the RRS1-R gene sequence data (NCBI Reference Sequence: NM_123894.3). The designing primer was performed at 350 bp (less conserved area) to see the expression in various tissues from inoculated red ginger, shampoo ginger, and big-white ginger.

![Figure 1. Wilt symptom on big-white ginger population (left); in red ginger (middle) and shampoo ginger population (right), ten days after inoculation.](image)

To observe the expression of the RRS1-R gene in various tissues (roots, stems, leaves), PCR was run at an annealing temperature of 45, 50, and 55°C, using four pairs of primers (forward and reverse primers), A = RRS1-R-GWF + RRS1-R-GWR; B = RRS1-R-GWF + RRS1-S-GWR; C = GTW-RRS1-For + GTW-RRS1-Rev; D = Expression RRS1-For + Expression RRS1-Rev. The amplification results showed that the four pairs of primers expressed annealing at 45°C, except for shampoo ginger, only the primer pair C (Figure 2).
Figure 2. The results of amplification of the RRS1-R gene for expression tests on roots, stems, leaves of Red Ginger (JM), and Shampoo ginger (LE) using four pairs of primers A = RRS1-R-GWF + RRS1-R-GWR; B = RRS1-R-GWF + RRS1-S-GWR; C = GTW-RRS1-For + GTW-RRS1-Rev; D = Expression RRS1-For + Expression RRS1-Rev. PCR program: Predenaturation 94 °C 7 min; Denaturation 94 °C 45 seconds; Annealing 45 °C 45 seconds; Extension 72 °C 2 minutes; Final Extension 72 °C 5 minutes.

The next step, the expression test was carried out using the primer pair of RRS1-Forward Expression and RRS1-Reverse Expression, run at the same annealing temperature of 45 C. The amplification results of all tested plant tissues showed high expression except for root tissue in shampoo ginger. The red ginger stem tissue results showed the brightest band intensity (rounded mark in Figure 3). This band was then purified and cloned on the pGEMT-Easy vector. Colony purification and PCR results from the Red Ginger stem DNA template are shown in Figure 4.

Figure 3. PCR results of DNA templates of Red Ginger (JM), shampoo ginger (LE), big-white ginger (JPB), and Somaklon (AC2), from roots (A), stems (B), and leaves (D) on annealing. 45°C.
The resulting colonies (no. 1 and 2) were then sequenced to obtain homologous genes for resistance to bacterial wilt. Unfortunately, the cloned DNA amplicon sequencing results showed that only red ginger origin amplicons could be analyzed with full-length cloned sequences (Figure 5). Thus, the shampoo ginger origins amplicons could not be analyzed due to the length of the amplified fragment of shampoo ginger and the frequency at which the locus fails to amplify completely.

Figure 4. DNA template purification of Red Ginger stems tissue (left) and PCR colony (right).

The results of DNA-cloned sequences from red ginger tissue after induction showed expression in stems. Another study revealed that tissue-specific expression of nine candidate genes related to defense response to R. solanacearum in susceptible Indian ginger and resistant mango ginger indicated that the rhizome tissue showed higher expression than leaf tissue for β-(1, 3)-glucanase. Meanwhile, HSP and Callose synthase showed higher expression in rhizome tissues [11]. Analysis of BLASTN (www.ncbi.nlm.nih.gov) full-length red ginger DNA-cloned after induction showed no homology to the RRS1 gene but homology to the Kafirin gene. Kafirin is a major storage protein in the grain such as sorghum and maize, which classified into four the α, β, γ, and δ and showed high homology with the equivalent zein proteins [12]. So far, there has been no data reporting the kafirin gene associated with plant resistance to diseases, especially bacterial wilt. These results indicate that different genes encoding resistance to bacterial wilt in ginger. Although the DNA samples tested were both from red ginger, the results of the BLASTN analysis (www.ncbi.nlm.nih.gov) full-length cloned DNA from red ginger leaves without induction were different. Full-length cloned DNA from red ginger without induction of R. solanacearum suspension homologous to Cacao swollen shoot virus polyprotein with E Value 2e-38 (Max Identity 62%), as shown in figure 6. Cacao swollen shoot virus (CSSV) is a member of the genus Badnavirus, family Caulimoviridae, naturally transmitted to cacao (Theobroma cacao) in a semi-persistent manner by multiple mealybug species [13]. This virus is recognized particularly in tropical plants. Cacao swollen shoot disease occurs in all the main cacao-growing areas of West Africa, where

GTGACCTGTTCGTTCAGCACAATTGATGACATGTGCTTTTTTACCCCTTTCAACTTTGTACAAAAAAGCAAGCTTAAATGAGGTCACCTAGCCGAACGACGCTCATCTGTATATACTGTA
GATCCTGTGTAGAGTCTTCAGAGAGAAGAGACTCTCCTCATTTGAAAGAAATTCATCCTGCAT
CCCTTTCTCTTGTCTGTATACCAAGAGATACATACATCATCTGATGATACATGATGCAGCA
TTGTATCCACCAATTTCATTTCCATCCAGGATTTCATATTTTCCATTCGCGCTAGGA
TTGATACCTTTGGGTGCCCCAGGTTTGAAGGCACCTGCCCCCTTGAGATACATGAT
AGAGACCATTATTGTCCGAAATAAACATTATGTCAGCTCCCTGTGTGTGCGAAAGGA
AGTGTCACTATTCATTATACACCATACTCTCTTGGATCGGATGACTCACTCTGATGATCTGATT
GTCGGGTCAGAAGGCGCTTTCCGCTACTGTGCAAGAATCCCTATGATACCAA
ACGACCCAGTTCTGTGTC

Figure 5. Full-length cloned DNA from red ginger amplified using a specific primer.
it has caused enormous damage. It is also present in Sri Lanka and Indonesia (Java and North Sumatra) [14].

DNA-cloned red ginger with or without induction showed their homology to the Kafirin gene and cacao swollen shoot instead of RRS1 gene, its probably due to the low-abundance differentially expressed cDNAs or genomic DNA fragments of those species. The RRS1 gene is discovered as encoding resistance to *R. solanacearum* (RSS1-R) in *Arabidopsis thaliana* [2, 3]; however, specific phosphorylation of RRS1-R reconfigures the complex, thereby activating the resistance reaction. Phosphorylation of proteins is a widely used regulatory mechanism in mammalian and plant immune signaling [15]. This circumstance might explain the misleading cDNA expression in the red ginger genome with or without pathogens induction. Further, the infection of microbial pathogens is followed by the delivery of virulence factors (effectors) that interfere with plant defenses. In plants, intracellular nucleotide-binding/leucine-rich repeat receptors (NLRs) detect specific effector interference and trigger immunity by an unknown mechanism [16].

While resistant varieties of gingers are not yet available, in the meantime, to overcome bacterial wilt attack in ginger cultivation, efforts can be taken by preparing disease-free superior seeds, land sanitation, and applying good agricultural practices, including early disease prevention and management.

Figure 6. Results of BLASTN analysis of cloned DNA sequences from Red Ginger without pathogen induction.
The above results indicate that the same plant has different responses to environmental manipulation, especially pathogen infection. Infected plant tissue mostly produced secondary metabolites as a response to infectious diseases. In tomatoes, about eleven metabolites consisting of amino acids, sugars, and organic acids were identified and presented at different concentrations in each cultivar. The defense mechanism in tomato plants against *R. solanacearum* was associated with the metabolites compound such as leucine and valine. Murti *et al.* found that leucine concentration is varied amongst tomato cultivars. They are predicted to be the most important compound responsible for contributing to the immune system and the defense systems in plants toward bacterial wilt [17]. Thus, the same pathogen might result in different responses associated with the host plant, including the biochemical mechanism inside the host plant tissue. Jiang *et al.* confirmed that higher tiller density in ginger affected higher ABA contents and higher expression levels of ABF4 as a response to high soil moisture. High soil moisture also leads to the presence of WAK 16 and WAK 32 genes which are determinant genes that attenuate resistance to *R. solanacearum* in ginger under high soil moisture [18].

Furthermore, the results of this study also suggest that the possible mechanism of plant resistance to bacterial wilt infection in ginger is in the stem tissue since the expression of cloned-DNA was observed most clear for stem tissue. Further study needed to be fulfilled.

4. Conclusion
The RRS1-R gene, encoding resistance to *Ralstonia solanacearum*, was not amplified from the total RNA of red ginger and shampoo ginger with or without pathogen induction. The results of DNA-cloned amplicon sequences isolated from DNA red ginger without induction, homologous to Cacao swollen shoot virus with E Value 2e-38 (Max Identity 62%). Meanwhile, DNA sequences cloned ginger and shampoo ginger after induction, homologous to the Kafirin gene group. Further research is needed to explore the library of resistance genes against bacterial wilt disease in Red Ginger and shampoo ginger with more effective methods such as Suppression Subtractive Hybridization (SSH). SSH could increase the probability of obtaining low-abundance differentially expressed cDNAs or genomic DNA fragments of ginger species and simplify the subtracted DNA library analysis.

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