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Full Length Article

Expression of *c-lysozyme* Gene in Transgenic Potatoes cv. Jala Ipam Against Bacterial Wilt Disease Caused by *Ralstonia solanacearum*

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Abstract

The objective of this study was to analyze the expression of the *c-lysozyme* gene in transgenic potatoes cv. Jala Ipam and their resistance to bacterial wilt disease cultivated in the isolated field. Inoculation of *Ralstonia solanacearum* Race 3 onto transgenic (JCL2 and JCL3) and non-transgenic (NT) plants derived from G0 tuber showed that all transgenic potatoes were more resistant to *R. solanacearum* than non-transgenic ones. JCL2 line was the most resistant line with disease frequency only 8.33%. Tuber production of JCL2 was 4 fold and JCL3 was 3.5 fold higher than non-transgenic line. The expression of *c-lysozyme* in transgenic lines was higher than that in non-transgenic line. JCL2 had the highest expression of the *c-lysozyme* gene compared to other lines. The relative expression of *lysozyme* gene in JCL2 was 4.83 fold to NT and 2.34 fold to JCL3. There was no expression of the *c-lysozyme* gene in non-transgenic line. It was found that higher expression of the *c-lysozyme* gene was correlated to higher resistance to bacterial wilt disease and to the higher tuber production. © 2020 Friends Science Publishers

Keywords: Bacterial wilt; *c-lysozyme*; Gene expression; *R. Solanacearum*; Transgenic potato

Intoduction

Potato plantations can be affected by diseases caused by bacterial pathogens, such as *Ralstonia solanacearum*, the agent of bacterial wilt disease (Genin and Denny 2012). This disease causes around 33% until 90% production loss (Elphinstone 2005). It is included in five major diseases that are commonly found in most production areas in Indonesia. *R. solanacearum* secretes EPS (exopolysaccharide) into the vascular tissue causing wilt disease. The infection process is carried out by producing several enzymes that hydrolyze the plant cell wall components to obtain nutrients and energy (Genin and Boucher 2002). *R. solanacearum* synthesizes endoglucanase (Schell 1987). Control of bacterial wilt has been carried out with the application of biocontrol using *Bacillus amyloliquefaciens* (Zhao *et al*. 2012), and conventional crossing with wild-type resistant to bacteria (Patil *et al*. 2012). Transgenic potato plants expressing lysozyme gene are resistant to *Erwinia carotovora* (Rivero *et al*. 2012).

Lysozyme is an enzyme that has the ability to degrade bacterial cell walls and cause bacteria lysis. It can be used to overcome the problem of bacterial diseases. The hydrolytic activity of lysozyme can degrade the structure of cell membranes and induce lysis of bacterial pathogen (Ibrahim *et al*. 2002). Some potato lines expressing *c-lysozyme* gene were resistant to *E. carotovora* subsp. *atroseptica*, *Streptomyces scabies*, *R. solanacearum*, *Pectobacterium carotovorum* subs. *carotovorum* (Serrano *et al*. 2000; Rivero *et al*. 2012).

The *c-lysozyme* gene has been successfully introduced into the genome of potato cv. Jala Ipam. *In vitro* assays showed that the transgenic potatoes containing *c-lysozyme* gene were resistant to *R. solanacearum* (Senjaya 2017). However, analysis of resistance in the field and *c-lysozyme* gene expression of these transgenic potato plants have not been studied yet. The objective of this study was to analyze the expression of the *c-lysozyme* gene in transgenic potatoes cv. Jala Ipam and their resistance to bacterial wilt disease in the isolated field.

Materials and Methods

Plant materials and bacterial strain

Sprouting G0 potato tubers of Jala Ipam transgenic lines, i.e. JCL2, JCL3, and non-transgenic (NT) line were used as

plant materials. *R. solanacearu*m race 3 from the Laboratory of Plant Bacteriology, Department of Plant Protection, IPB University, were used for inoculation.

R. solanacearum infection assays

The experiment was conducted in the isolated field using 3 lines, and 3 replications in randomized block design, so this experiment was composed of 9 plots. One plot contained 4 plants. Bacterial inoculation was applied to 45 days-old plants. *R. solanacearum* was cultured in nutrient broth until OD_{600nm} 0.7 or equivalent to 1.2 x 10^9 cells/mL. Inoculation was carried out by spraying bacterial suspension throughout the leaf and stem surface. The resistance level of plants was evaluated according to the procedure described by Thaveechai *et al*. (1989) based on the frequency of the disease.

Total RNA isolation

Total RNA was isolated from young leaves using TRIzol® Reagent (Invitrogen). Potato leaves in the presence of liquid nitrogen were ground in a mortar to become powder. The powder was put in 1.5 mL micro tube, mixed with 800 µL of the TRIzol solution. The suspension was mixed with 200 µL of chloroform, shake thoroughly and incubated at room temperature for 3 min. The suspension was centrifuged at 10.000 rpm for 10 min at 4°C. The supernatant was mixed with isopropanol as much as 1 x supernatant volume, incubated for 10 min at room temperature and centrifuged at 10,000 rpm for 10 min at 4°C. The pellet was washed by adding 500 μL ethanol 75% and centrifuged at 10,000 rpm for 5 min at 4°C. After drying, the pellet was mixed with 15 µL DEPC-treated H_2O and incubated at 60^{o}C for 10 min.

Total cDNA synthesis

Total RNA was treated with DNAse by mixing 10 mL total RNA, 1.1 μL DNAse buffer (10x), and 0.2 μL DNAse. The suspension was incubated at room temperature for 5 min, and then mixed with 1 μL EDTA. cDNA synthesis was carried out by using iScript™ cDNA Synthesis Kit (Bio-Rad, US) in PCR micro tube, by mixing 1 μL total RNA, 2 μL buffer (5x) iScript, 0.5 μL reverse transcriptase iSCript and 6.5 μL nuclease free water in a total volume of 10 μL and then incubated at 25°C for 5 min, 42°C for 30 min and 85°C for 5 min. PCR to part of actin gene was applied to evaluate the quality of total cDNA by using specific actin primers, Tact-qF (5'-ACA TCG TCC TTA GTG GTG GA-3'), and Tact-qR (5'-GTG GAC AAT GGA AGG ACC AG-3'), located at exon 3 and exon 4 of actin gene, respectively. The condition of PCR was pre-PCR at 95°C for 5 min, denaturation at 95°C for 30 sec, annealing at 55°C for 45 sec and extension at 72°C for 1 min and 35 cycles.

Analysis of gene expression with quantitative real-time PCR

A primer pair of Lys114-F (5'-TAT GAA GCG TCA CGG ACT TG-3') and Lys359-R (5'-TTC ACG CTC GCT GTT ATG TC-3') (Mustamin 2017) was used to amplify the *c-lysozyme* gene and Tact-qF, and Tact-qR was used to amplify the actin gene. PCR reaction was composed of 1 μL cDNA (50 ng), 5 μL SsoFast ™ Eva Green® Supermix, 0.25 μL forward primer, 0.25 μL reverse primer and 3.5 μL nuclease free water. The program of qRT-PCR was pre-PCR at 95°C for 30 sec, followed by 45 cycles with denaturation at 95°C for 5 sec, annealing at 58°C for 10 sec and extension at 72°C for 10 sec.

Data analysis

Data was analyzed using analysis of variance and Tukey's test with SPSS Version 16. The gene expression was analyzed using CT (cycle threshold) comparison method and relative expression ($2^{-\Delta\Delta CT}$) (Livak and Schmittgen 2001). The relative expression of genes were calculated by using formula as follow: $\Delta CT_{JCL} = CT_{Lys} - CT_{Act}$; $\Delta CT_{NT} = CT_{Lys} - CT_{Act}$; $\Delta\Delta CT = \Delta CT_{JCL} - \Delta CT_{NT}$, where CT is the number of cycles for the fluorescence signal to pass the threshold. ΔCT is the difference of CT between the target gene (*c-lysozyme*) and the standard gene, i.e. actin gene. CT_{Lys} is CT value of the *c-lysozyme* gene and CT_{Act} is CT value of the *actin* gene. JCL is transgenic lines and NT is non-transgenic line. $\Delta\Delta$CT is the difference between ΔCT transgenic and ΔCT non-transgenic.

Results

Resistance of potato plants to *R. solanacearum*

Bacterial wilt disease was found in all non-transgenic lines with wilt symptoms on the leaves and stems. The basal of the stem and all plant organs became brown. On the other hand, the transgenic lines, *i.e*., JCL2 and JCL3, were still alive, indicated by green leaves and the stems (Fig. 1).

Among the three lines, JCL2 was the most resistant line to bacterial wilt disease. The frequency of the disease of JCL2 was only 8.33% (Table 1). Although some transgenic plants were infected, these plants died later than non-transgenic one. When potato plants were harvested at 95 days old, the stems of transgenic plants were still fresh and green while the stems of non-transgenic ones were dry and brown.

Potato tuber production after infection of *R. solanacearum*

After inoculation by *R. solanacearum*, the tuber production of three lines at 90 days after planting, was significantly different ($P \leq 0.05$). Based on Tukey's Test ($P \leq 0.05$),

JCL2 line had the highest production. The weight of tuber of transgenic lines was higher than non-transgenic ones, whereas the number of tuber of JCL3 was not significantly different to non-transgenic line (Table 2). The production of JCL2 and JCL3 was 4 fold and 3.5 fold, respectively, to non-transgenic lines. In general, the size of tubers of non-transgenic lines was smaller than transgenic lines (Fig. 2). Bacterial wilt caused non-transgenic potato plant unable to continue to grow limiting the number and the size of tubers production.

Table 1: The degree of resistance of potato plants to *Ralstonia solanacearum*

Lines	Number of Plants	Incidence of disease	Frequency of Disease (%)	Degree of Resistance
NT	12	12	100	Sensitive
JCL2	12	1	8.33	Resistant
JCL3	12	3	25	Rather Resistant

Table 2: Tuber production per plant after *R. solanacearum* inoculation

Lines	Production of tuber per plant	
	Weight (g)	Number
NT	29.36[a]	2.75[a]
JCL2	119.73[b]	5.25[b]
JCL3	105.22[b]	4.00[a]

Note: the numbers followed by different letters in the same column were significantly different at the 95% confidence level

Fig. 1: Symptom of bacterial wilt disease 4 weeks after inoculation

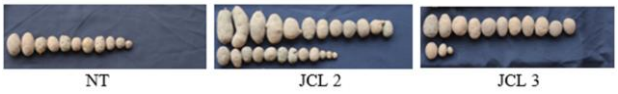

Fig. 2: The potato tubers produced by one plot at 95 days after planting

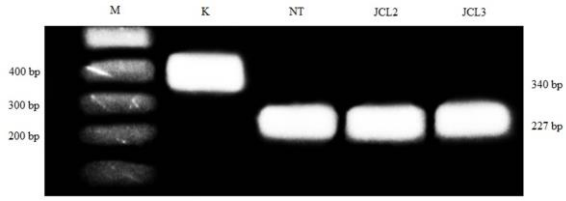

Fig. 3: PCR products by using Tact-qF and Tact-qR primers. M: marker of 1 Kb DNA Ladder, K: Genomic DNA, NT: cDNA of non-transgenic line, JCL2 and JCL3: cDNA of transgenic lines

Expression of *c-Lysozyme* gene

Total cDNA was successfully synthesized from total RNA as template. To confirm that this cDNA did not contain genomic DNA, we carried out PCR to amplify the region between third and fourth exons of actin gene by using Tact-F and Tact-R primers. PCR with this pair of primer resulted 227 bp of amplicon (Fig. 3). When these primers amplified the genomic DNA between third and fourth exon of actin, the PCR resulted 340 bp. This result indicated that total cDNA was successfully synthesized without contamination from the genomic DNA.

PCR by using a primer pair of Lys114F and Lys359R showed that 245 bp cDNA of transgenic lines was amplified, but there was no amplification in cDNA of non-transgenic line (Fig. 4). This result showed that *c-lysozyme* gene was expressed in transgenic lines and there was no expression in non-transgenic line.

Analysis of quantitative expression of *c-lysozyme* gene showed that there was a significant difference among the potato lines ($P \leq 0.05$). Relative expression of *c-lysozyme* in transgenic lines was higher than in non-transgenic line. The highest expression of *c-lysozyme* was found in JCL2 line, followed by in JCL3. Relative expression of *c-lysozyme* in JCL2 line was 4.83 fold to NT and 2.34 fold to JCL3. On the other, the JCL3 line had an expression of 2.06 fold to NT (Fig. 5).

Discussion

There was a correlation between the level of expression of *c-lysozyme* gene and the level of resistance to bacterial wilt disease and the level of tuber productivity of plants. JCL2 plants expressed the highest *c-lysozyme* gene and the most resistance to *R. solanacearum*. This transgenic line had the highest tuber productivity. On the other, non-transgenic plants did not contain *c-lysozyme* gene, therefore there was no expression of *c-lysozyme* gene. Since there is no expression of *c-lysozyme* gene, non-transgenic plants are sensitive to *R. solanacearum*.

Expression of *c-lysozyme* gene in JCL2 was higher than in JCL3, and JCL2 more resistant to *R. solanacearum* than JCL3. This result indicated that the level of *c-lysozyme* gene expression was closely related to the level of resistance to *R. solanacearum*. The higher expression of the *c-lysozyme* gene, the higher was the resistance to bacterial wilt disease. As an antimicrobial enzyme, lysozyme can degrade peptidoglycan of gram-positive and gram-negative bacteria resulting bacterial lysis (Serrano *et al*. 2000).

The different expression of *c-lysozyme* gene between JCL2 and JCL3 may be caused by the different position of insertion of *c-lysozyme* gene in the potato genome (Düring *et al.* 1993; Serrano *et al.* 2000; Dong *et al.* 2008). The different position of gene insertion was caused by the random transfer and insertion of the gene into the plant

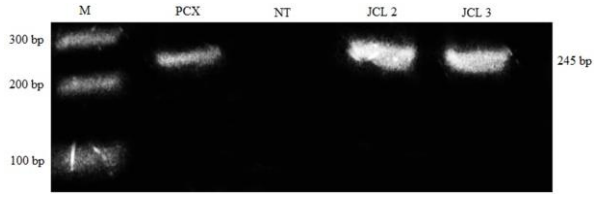

Fig. 4: PCR products by using a primer pair of Lys114F and Lys359R, and total cDNA as template. M: marker of 100 bp DNA Ladder, K: *lysozyme* gene inserted in pCX plasmid, NT: cDNA of non-transgenic line and JCL2 and JCL3: cDNA of transgenic lines

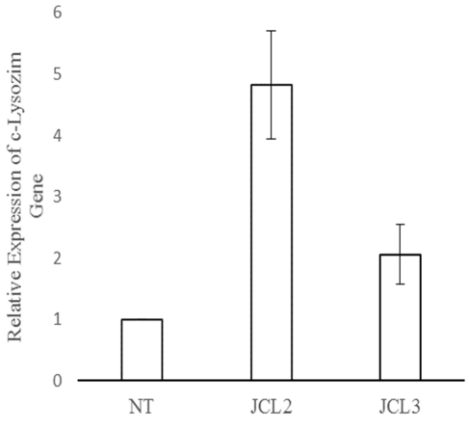

Fig. 5: The relative expression of *c-lysozyme* gene in transgenic lines (JCL 2 and JCL 3) compared to non-transgenic potatoes cv. Jala Ipam

genome mediated by *Agrobacterium tumefaciens*.

Increasing the resistance to bacterial wilt can keep plant to grow till the harvest. It is therefore, more resistant plant has more tuber productivity. JCL2 is most resistant to bacterial wilt disease compared to other lines, so it has highest tuber productivity.

The infection process of *R. solanacearum* can be divided into 3 stages, namely root colonization, plant root cortical infection and xylem penetration. Root colonization is described by the formation of colonies around the roots. The bacteria can penetrate the plant through a physical or natural wound (Vasse *et al*. 1995). The bacteria use pili to attach the root (Sequeira 1985) and flagella to penetrate into plant tissues (Tans-Kersten *et al*. 2001). At the stage of the plant root cortical infection, *R. solanacearum* begins to infect the root by forming colonies in the intercellular space (Vasse *et al*. 1995) and secreting enzymes to degrade plant cell walls (Schell 2000). At the xylem penetration stage, bacteria penetrate from the cortex to the xylem through the endodermic tissue (Saile *et al*. 1997). This process was stopped by lysozyme when bacteria entered the intercellular space. Lysozyme can be secreted into the intercellular space then degrades bacteria that enter the plant (Düring 1993). The construct of the *c-lysozyme* gene in this study did not use a peptide signal (Senjaya 2017), so lysozyme remains in the cytosol and degrades every bacterium that enters into the plant cells. This condition caused lysozyme to stop the infection of *R. solanacearum* at the third stage of infection, i.e. xylem penetration. When the bacteria penetrate into the xylem, the bacteria will penetrate into the cells in the cortex and endodermic tissue (Saile *et al*. 1997). Another mechanism that causes lysozyme exit from the cytosol is due to the change in membrane permeability caused by bacteria (Chen 2014; Fatima and Senthil-Kumar 2015). When the cells infected by bacteria, the nutrient from the cytosol can be secreted to the intercellular space (Wang *et al*. 2012; Chen 2014; Fatima and Senthil-Kumar 2015). This mechanism allows the *c-lysozyme* released into the intercellular space, even though the construct does not have a signal peptide.

Lysozyme is a protein that is very important for defense against bacterial infections (Magnadottir 2006). *c-lysozyme* is a type of c-type lysozyme (chicken type) (Irwin and Gong 2003) found in both vertebrates and invertebrates (Zhao *et al*. 2007). The type of lysozyme gene introduced in this study was *c-lysozyme* type isolated from chicken. The *c-lysozyme* gene is composed of four exons. Exon 2 encodes amino acids at 28–82 which are involved in residual catalysis and binds the C, D, E and F rings of the oligosaccharide substrate. Ekson 3 encodes amino acids at 82–108 which enhances recognition and specifications on the substrate. Exon 1 and 4 encode signals for translation of mRNA but are not directly involved in catalysis activities (Jung *et al*. 1980). Lysozyme breaks the β-1,4 bond between N-acetylmuramic and N-acetylglucosamine in peptidoglycan. It causes bacterial lysis and bacterial death (Osserman and Lawlor 1966).

To examine the presence of the *c-lysozyme* gene in cDNA potatoes cv. Jala Ipam, qRT-PCR products were electrophoresed on 2% agarose gel. There was no band in non-transgenic lines, but a band of 245 bp was found in the transgenic lines and pCX (plasmid control) (Fig. 4). It proved that the resistance of potato lines to bacterial wilt caused by *R. solanacearum* was due to the presence of the *c-lysozyme*.

Conclusion

The higher expression of *c-lysozyme* gene results into more resistance to bacterial wilt disease leading to higher tuber productivity. JCL2 line had the highest expression of *c-lysozyme* gene, highest resistance to bacterial wilt disease and highest tuber productivity.

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