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Obtaining of transgenic potato (*Solanum tuberosum* L.) cultivar IPB CP3 containing *LYZ-C* gene resistant to bacterial wilt disease

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ABSTRACT Bacterial wilt caused by *Ralstonia solanacearum* is one of the most important bacterial diseases in potato production. This study aimed to obtain the transgenic potato (*Solanum tuberosum* L.) cultivar IPB CP3, containing *LYZ-C* gene encoding for lysozyme type C, resistant to bacterial disease caused by *R. solanacearum*. Genetic transformation using *Agrobacterium tumefaciens* LBA4404 to 124 internode explants resulted in the transformation efficiency of about 47.58% with a regeneration efficiency of approximately 30.51%. Gene integration analysis showed that 16 clones were confirmed as transgenic clones containing the *LYZ-C* gene. Analysis of resistance to *R. solanacearum* of three transgenic clones showed that all three transgenic clones were more resistant than a non-transgenic one. This result showed that the *LYZ-C* gene integrated in the genome of transgenic potato increased the resistance of potato plants to *R. solanacearum*. We obtained two transgenic clones considered resistant to bacterial wilt disease.

KEYWORDS Bacterial wilt; genetic transformation; lysozyme; potato; transgenic

1. Introduction

Potato is the third major food crop based on total consumption in the world after rice and wheat (FAOSTAT 2020). In Indonesia, consumption of potatoes tends to continue to increase, especially in the form of processed potatoes such as french fries and chips. However, potato production still faces several obstacles, mainly due to the availability of low-quality potato seeds, disease infections, and unfavorable environmental conditions. Potato disease can quickly spread and develop since potato is propagated vegetatively (Davidson and Xie 2014). Bacterial wilt disease caused by *Ralstonia solanacearum* is the most common disease to attack potato plants. *R. solanacearum* is reported to attack more than 250 plant species; most of the hosts belong to the *Solanaceae* and *Musaceae* families (Charkowski et al. 2019).

Possible actions to control the infection and the spread of pathogens in potato are to use disease-resistant cultivars through the fusion of protoplasts between species (Fock et al. 2000) and the introduction of disease resistance genes (*R* genes) to induce innate immune responses (Gururani et al. 2012). Lysozyme is an enzyme with bacteriolytic activity capable of degrading peptidoglycan, a constituent of bacterial cell walls (Yon-Kahn 1996). A gene encoding for lysozyme has been incorporated into many plants to increase the resistance to pathogenic infections. The introduction of the *T4 lysozyme* gene into tall fescue grass (*Festuca arundinacea* Schreb.) succeeded in obtaining transgenic grass plants resistant to *Rhizoctonia solani* and Magnaporthe grisea (Dong et al. 2008). The *LYZ-C* gene encoding c-type lysozyme was also introduced to potato cultivar Desiree, and the obtained transgenic potato plants were resistant to *Erwinia carotovora* subsp. *atroseptica* (Serrano et al. 2000).

Transgenic potato cv Jala Ipam harboring *LYZ-C* gene under the control of 35S CaMV promoter was also resistant to *R. solanacearum in vitro* (Senjaya 2017) and the field condition (Alfian et al. 2020). Although these cultivars are very promising to be applied, potato cv Jala Ipam is sterile and tetraploid. Therefore this clone is not able to be a donor of *LYZ-C* gene to other potato cultivars through conventional breeding by sexual crossing. Thus, this study aimed to introduce the *LYZ-C* gene under the control of

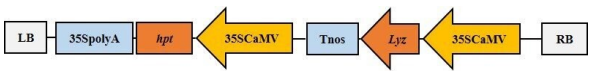

FIGURE 1 Map of the T-DNA region of the pCXSN-*Lyz* plasmid carrying *LYZ-C* gene under the control of 35S CaMV promoter and terminator of nopaline synthase, and the cassete of *hpt* gene as selectable marker (Senjaya 2017).

35S CaMV promoter into another superior potato cultivar, IPB CP3, to increase its resistance to *R. solanacearum* as a causal agent of bacterial wilt disease.

2. Materials and Methods

2.1. Plants Materials and Agrobacterium tumefaciens Propagation

IPB CP3 cultivar was propagated in vitro on an MS-based medium (Murashige and Skoog 1962). The cuttings were grown in a culture room at 24-25 °C for 3-4 weeks with a photoperiod of 18/6 h and 2,000-3,000 lux light intensity. *Agrobacterium tumefaciens* strain LBA4404 carrying the recombinant plasmid pCXSN-Lyz Figure 1 was used to transform the potato genetically. *A. tumefaciens* was cultured in LB (Luria Bertani) liquid medium supplemented with 50 mg/L of kanamycin, 50 mg/L of hygromycin, and 100 mg/L of streptomycin in the dark condition at room temperature for 15-18 h until the optic density at 600 nm was about 0.5. The suspension of *A. tumefaciens* was centrifuged at 10,000 rpm for 10 min. The pellet was resuspended in inoculation medium (MS medium containing 16.0 g/L of glucose, 2.0 mg/L of 2,4-D, 0.8 mg/L of trans-Zeatin) until OD_{600} was about 0.3.

2.2. Potato Transformation

Internodes with size of 0.5-1 cm were used as explants and grown on solid preculture medium (PC) (MS medium containing 2 mg/L 2,4-D, 0.8 mg/L trans-Zeatin, 40.0 mg/L acetosyringone) for 2 days. Explants were put into a liquid inoculation medium containing *A. tumefaciens* and shake softly for 10 min at room temperature. The explants were then dried on sterile tissue paper for 5 min and grown on solid co-cultivation medium (CO) (MS medium containing 16.0 g/L of glucose, 2.0 mg/L of 2,4-D, 0.8 mg/L of trans-Zeatin) for three days in the darkroom. The explants were rinsed three times by sterile distilled water containing 200 mg/L cefotaxime then dried on sterile tissue paper for 5 min. Furthermore, explants were grown on solid callus induction medium (CI) (MS medium supplemented by 1.0 mg/L of IAA, 0.5 mg/L of GA3, 0.8 mg/L of trans-Zeatin, 100.0 mg/L of cefotaxime) for three weeks. Callus was subcultured every two weeks in a selection medium containing 10 mg/L hygromycin until it regenerated the shoots. The shoots were further propagated by subculturing every three weeks in MS medium containing hygromycin with a gradual increase as much as 10 mg/L, 20 mg/L, and 30 mg/L.

2.3. Molecular Analyses of Transformed Potato Plants

Genomic DNA of potato was isolated by the CTAB method (Suharsono 2002) using a 2% CTAB buffer. Integrated transgenes in transgenic plants were analyzed by PCR using primer pair of Lyz114-F (5'-TAT GAA GCG TCA CGG ACT TG- 3') and NosT2-R (5'-GAA TCC TGT TGC CGG TCT TGC G-3'). The PCR was carried out by the condition of pre-denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 54 °C for 45 s, and extension 72 °C for 1 min, and post-PCR at 72 °C for 5 min. PCR results were electrophoresed in 1% (w/v) agarose gel, 100 V for 28 min. 1 kb DNA ladder (*Thermo Fisher Scientific*, USA) was used as a molecular weight marker.

2.4. Bacterial Inoculation Assays

In vitro inoculation assays were carried out using the bacteria *R. solanacearum* as described by Habe (2018). Plants were grown in the jar containing 40 mL vermiculite and 30 mL MS liquid medium. Four weeks old plants were inoculated by 200 µL of bacterial suspension with a concentration of $2x10^9$ CFU/mL. Incubation was carried out at 25-28 °C. Disease symptoms were observed at 10 d after inoculation. The frequency of disease was calculated using a formula as follows.

$$Disease\ frequency = \frac{Number\ of\ non\ resistance\ plants(n)}{Total\ plants(N)} \times 100\% \quad (1)$$

The level of plant resistance was determined as described by Thaveechai et al. (1989), where 0-20% of disease frequency is considered as resistant (R), 21-40% is moderately resistant (MR), 41-60% is moderately susceptible (MS), and 61-10% is susceptible (S).

3. Results and Discussion

3.1. Transformation of Potato Plants

The *LYZ-C* gene was successfully inserted into the genome of the potato cultivar IPB CP3 with *Agrobacterium*-mediated transformation. *A. tumefaciens* is widely used to introduce genes because naturally, it can efficiently transfer and integrate stably DNA fragments contained in the T-DNA region into the genome of the host plant (Hwang et al. 2017). The process of genetic transformation of potato cv IPB CP3 is presented in Figure 2.

The stem segment explants (Figure 2a) used in this study began to form a callus at two weeks after growing in CI medium. Callus was formed starting from the ends of the stem segments and it grew to cover the entire surface of the explants (Figure 2b). Callus began to regenerate to form shoots at 4 weeks of age. The stem-segment explants developed callus and then regenerated to form shoots on a medium containing 1 mg/L of IAA hormone, 3 mg/L of trans-Zeatin hormone, and 0.5 mg/L of GA3 hormone. Masekesa et al. (2016) explained that the success of callus induction and regeneration depends on the genotype of the explants and the right combination of concentrations the number of hormones used. The cytokinin hormone in the form of trans-Zeatin is able to induce both callus and shoot formation (Park et al. 2003).

This study used *in vitro* precultured explants of internodes (Figure 2). During growth in PC medium, most of the explants developed to tissue swelling. McHughen et al. (1989) showed that preculture prior to inoculation with *A. tumefaciens* increase the production of transgenic plants.

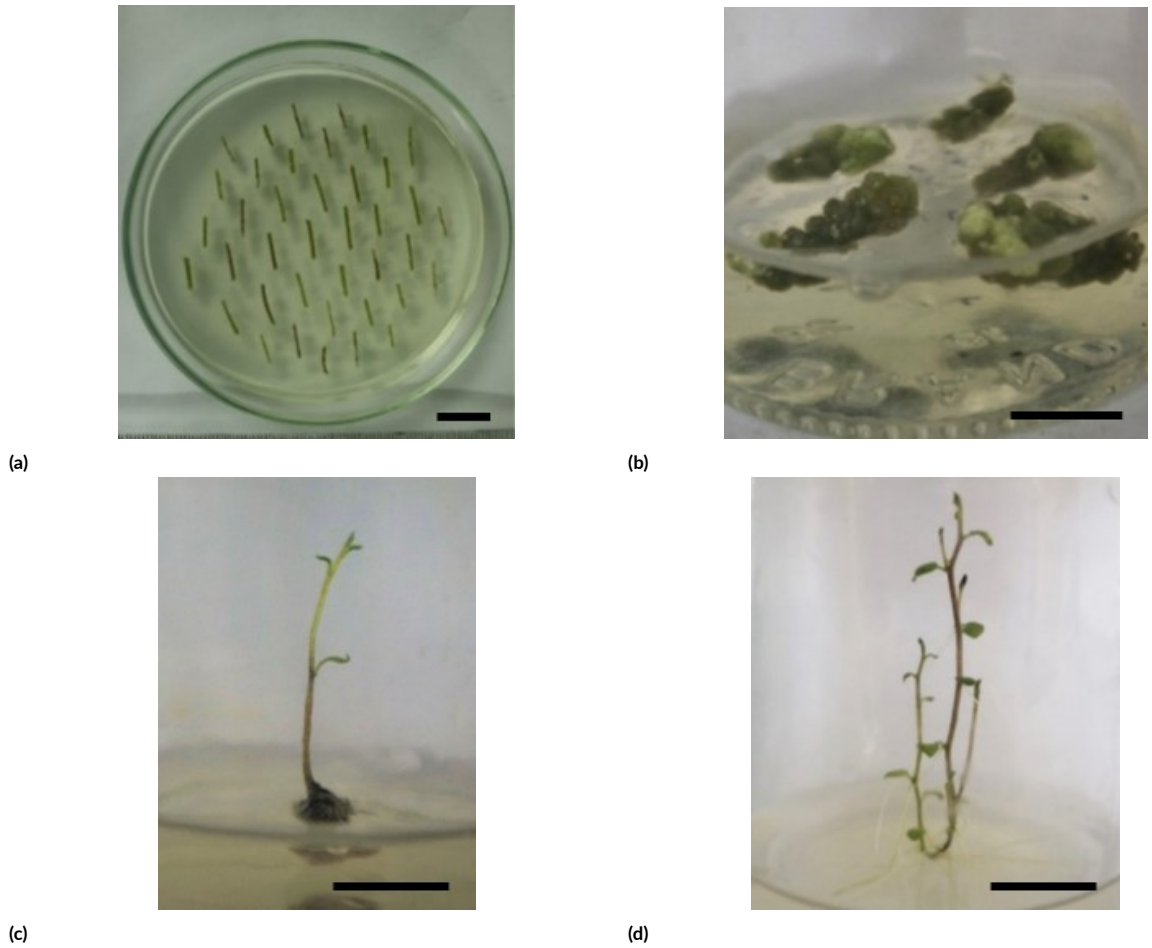

FIGURE 2 The process of genetic transformation of the potato cultivar IPB CP3 by the *LYZ-C* gene. (a) Explant of internode on preculture medium, (b) Callus formation, (c) Shoot regeneration, and (d) Putative transgenic plants. Bar= 1 cm.

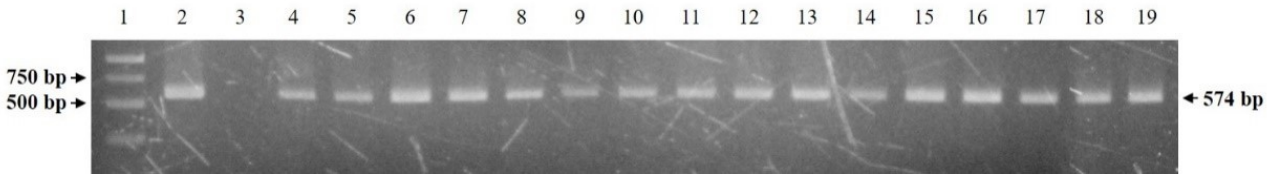

FIGURE 3 The amplification of the *LYZ-C* gene (574 bp). 1: 1kb DNA ladder marker, 2: DNA plasmid pCXSN-*Lyz*, 3: Non-transgenic of potato cultivar IPB CP3 and 4-19: Transgenic of potato cultivar IPB CP3.

Furthermore, Sangwan et al. (1992) showed that preculture treatment increased the number of competent cells to be transformed by *A. tumefaciens*. The treatment of explants on PC medium played a role in adjusting the stress conditions due to co-cultivation with *Agrobacterium*. To induce the process of gene transfer from *A. tumefaciens* into plant explants, the co-cultivation medium was supplemented with acetosyringone 40 mg/mL. Acetosyringone phenolic compounds increased the efficiency of transformation in plants with *Agrobacterium*-mediated transfor-

TABLE 1 The efficiency of transformation and regeneration of the potato cultivar IPB CP3 based on the selection using 10 mg/L hygromycin.

Repitition	Total Explant	Total Resistant Callus	Total Regenerated Callus	Transformation Efficiency (%)	Regeneration Efficiency (%)
1	127	53	16	41.73	30.18
2	123	61	25	49.59	40.98
3	122	63	13	51.63	20.63
Total	372	177	54	-	-
Averages	124	59	18	47.58	30.51

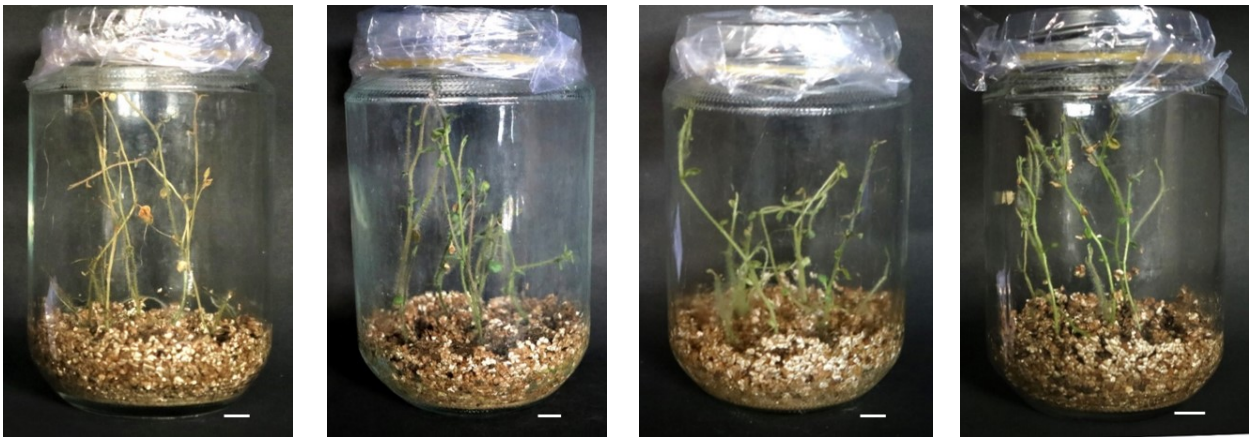

FIGURE 4 Morphology of plants innoculated by *R. solanacearum.* (a) Non-transgenic potato cultivar IPB CP3, (b) CP3lyz1 clone, (c) CP3lyz2 clone, (d) CP3lyz6 clone. Bar= 1 cm.

mation, such as in ornamental plants *Eustoma grandiflorum* (Nakano 2017), and in plant species of *Melastoma malabathricum* and *Tibouchina semidecandra* plants (Yong et al. 2006).

The results showed that some explants were able to keep growing to form a callus and regenerate to develop shoots on a selection medium containing 10 mg/L hygromycin. However, some explants also slowly turned into brown color (browning) and underwent necrosis. In general, browning is caused by the phenolic compound secreted by the wounded tissue (Leng et al. 2009). From three experiments, the transformation efficiency based on the selection using 10 mg/L hygromycin was ranged between 41.73% and 51.63%, with an average of 47.58% (Table 1). Based on the number of hygromycin-resistant calli, the efficiency of regeneration ranged from 20.63% and 40.98%, with an average of 30.51% (Table 1). The transformation efficiency in this study was higher than those of transformation of potato cultivar Jala Ipam by *MmPMA* gene (Farhanah et al. 2017) and potato cultivar IPB CP1 cultivar by *Hd3a* gene (Gea et al. 2017). The explants infected with *A. tumefaciens* but not containing pCXSN-*Lyz*, enabled to form callus in the medium without hygromycin. However, these calli were not able to survive in the selection medium containing 10 mg/L hygromycin. This phenomenon shows that 10 mg/L hygromycin was effective for the selection of transgenic calli. The efficiency of regeneration in this study was higher than the regeneration efficiency obtained by Gea et al. (2017) and Farhanah et al. (2017).

Shoots regenerated from callus grew on the selection medium (Figure 2c), separated from the callus in order to form roots and for propagation (Figure 2d). A total of 31 independent shoots regenerated from resistant calli. These shoots were multiplied in a selection medium where the hygromycin concentration was gradually increased to 30 mg/L. These shoots are called putative transgenic clones. Among these 31 clones, we chose 16 transgenic clones that had the fastest growth to be analysed.

3.2. Molecular Analysis of the Transgenic Potato Plants

PCR analysis of 16 clones of putative transgenic plants with Lyz114-F and NosT2-R primers showed that all clones contained *LYZ-C* transgene and nopaline synthase terminator (tNOS) with a fragment size of 574 bp (Figure 3). Similarly, the amplification results were obtained by using the pCXSN-*Lyz* plasmid as a DNA template. This plasmid was used as a positive control. Conversely, the non-transgenic (NT) plant did not produce these amplicons as expected because it was used as the negative control (Figure 3). This result proved that the *LYZ-C* gene was successfully integrated into the transgenic plants.

3.3. Bacterial Resistance Analysis of the Transgenic Potato Plants in vitro

The resistance assay against *R. solanacearum* was carried out on three selected clones with the fastest growth, namely CP3lyz1, CP3lyz2, and CP3lyz6 clones from a total of 16 clones of transgenic plants. One non-transgenic plant cultivar IPB CP3 was included in this experiment as a control. After inoculation by *R. solanacearum*, the transgenic clones could survive with different levels of resistance, whereas the non-transgenic one showed the symptom of wilt disease (Figure 4). The sign of the disease was indicated by the change of the color of leaves, from green to yellow, then to brown, followed by a broken stem. The wilt in plants infected by *R. solanacearum* is caused by a disfunction of the plant's vascular system. *R.*

TABLE 2 The level of resistance of plant to *R. solanacearum*.

Klon	Total Inoculated Plant	Total non-resistant Plant	Disease Frequency (%)	Resistence Level*
CP3 NT	30	30	100.0	S
CP3lyz1	30	6	20.0	R
CP3lyz2	30	11	36.7	MR
CP3lyz6	30	5	16.7	R

solanacearum reproduces and colonizes the xylem vessels before blocking the water and mineral transport (Lowe-Power et al. 2018) and causing plants to wilt.

The bacterial wilt disease frequency of clones was ranged from 16.7% to 100.0%. Based on Thaveechai et al. (1989), CP3lyz1 and CP3lyz6 clones were resistant to *R. solanacearum*, whereas CP3lyz2 clones were moderately resistant to *R. solanacearum*. Meanwhile, all non-transgenic potato cultivar IPB CP3 plants were infected by *R. solanacearum*, therefore this variety was sensitive to *R. solanacearum* (Table 2). Transgenic clones are more resistant to R. solanacearum than the non-transgenic one, possibly because transgenic clones are able to synthesize lysozyme, while non-transgenic clones are unable to synthesize lysozyme. CP3lyz1 and CP3lyz6 clones were resistant to R. solanacearum, possibly because the two clones were able to overexpress the *LYZ-C* gene. Both clones, CP3lyz1 and CP3lyz6, were more resistant to *R. solanacearum* than the other transgenic clone, possibly because the expression of the *LYZ-C* gene in both clones was higher than in the other clone. Quantitative *LYZ-C* gene expression in the transgenic clones has to be investigated to know the expression level of *LYZ-C* gene in transgenic clones.

The lysozyme encoded by the *LYZ-C* gene is capable to cleave the β-1,4-glycosidic bond between N-acetyl-D-muramic acid (MurNAc) and N-acetyl-D-glucosamine (GlcNAc) of peptidoglycan, the cell wall bacteria component (Yon-Kahn 1996). Therefore lysozyme can kill the bacteria, including *R. solanacearum*, by degrading their cell wall.

The difference in the resistance levels of transgenic plants containing the *LYZ-C* gene under the control of the same 35S CaMV promoter could be caused by the different expression level of *LYZ-C* gene. Differences in transgene expression level in transgenic plants containing the same transgene are common phenomena (Kohli et al. 2006). The level of gene expression among clones of the transgenic potato cultivar Jala Ipam containing the same *LYZ-C* gene varied and the level of the resistance against *R. solanacearum* was correlated to the expression level of *LYZ-C* gene (Alfian et al. 2020). Differences in transgene expression levels can be caused by the copy number of transgene insertion, the site of transgene insertion, RNA silencing, and the regulators for transgene expression (Butaye et al. 2005). T-DNA can be integrated into the plant genome randomly and distributed throughout all parts of the chromosome (Ko et al. 2018). Therefore it can be inserted in the encoding and non-coding regions. Random insertion can affect the level of gene expression in transgenic plants due to the effect of copy number insertion and the impact of transgene position.

4. Conclusions

LYZ-C gene was successfully integrated into the genome of transgenic potato cv IPB CP3. *In vitro* resistance assay to *R. solanacearum* of three transgenic clones showed that transgenic clones were more resistant to *R. solanacearum* than non-transgenic ones. These results indicate that the *LYZ-C gene* integrated in the transgenic potato cultivar CP3 IPB could increase the resistance to bacterial wilt disease caused by *R. solanacearum*.

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Authors' contributions

P, S, AT designed the research. P carried out the laboratory work. P, S analyzed the data. P wrote the manuscript. S, AT revised the article and approved the final version of the manuscript.t.

Competing interests

The author declare that they have no competing interest.

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