Indonesian Journal of Agricultural Science Vol. 16 No. 2, October 2015: 51-58

GENETIC STABILITY ANALYSIS OF *RB* GENE IN GENETICALLY MODIFIED POTATO LINES TOLERANT TO *Phytophthora infestans*

Analisis Stabilitas Genetik Gen RB *pada Tanaman Kentang Transgenik Tahan terhadap* Phytophthora infestans

Edy Listanto, Eny Ida Riyanti, Tri Joko Santoso, Toto Hadiarto and A. Dinar Ambarwati

Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development
Indonesian Agency for Agricultural Research and Development
Jalan Tentara Pelajar No. 3A, Bogor 16111, West Java, Indonesia, Phone +62 251 8337975, Fax. +62 251 8338820
E-mail: bb_biogen@litbang.pertanian.go.id
Corresponding author: edy_listanto@yahoo.com

Sumbitted 29 December 2014; Revised 30 April 2015; Accepted 30 April 2015

ABSTRACT

Development of potato cultivars with high levels of broad spectrum resistance is a key long-term management strategy against late blight disease caused by *Phytophthora infestans*. Six progeny lines of hybridization between transgenic potato Katahdin SP951 with non-transgenic Granola and Atlantic were selected based on agronomical characteristics and resistance to late blight disease. The study aimed to analyze the number of insertions and stability of inserted *RB* gene in the transgenic potato lines. The research was carried out through plant DNA extraction, southern blot analysis and polymerase chain reaction (PCR). Southern blot analysis was used to detect the number of inserts integrated into potato genome, while PCR analysis was used to detect stability of *RB* gene from generation to generation. The results showed that the progenies obtained from hybridization between Atlantic and transgenic Katahdin SP951 (lines No. 20 and 27) and between Granola and transgenic Katahdin SP951 (line No. 69) contained one copy number of *RB* gene, according to the probing of *npt*II. The result is similar to that of inserted *RB* gene found in the parental transgenic Katahdin SP951. The presence of *RB* gene in four different generations (G_0, G_1, G_2 and G_3) showed stable integration of the gene into the plant genome. The single copy number of *RB* gene will repress the occurrence of silencing gene expression. The stability analysis of *RB* gene can determine that the gene is still present in plant genome after several generations.

[***Keywords*:** Transgenic potatoes, genetic stability, *Phytophthora infestans*, *RB* gene]

ABSTRAK

Pengembangan tanaman kentang transgenik tahan terhadap penyakit hawar daun yang disebabkan oleh Phytophthora infestans *berspektrum luas merupakan salah satu strategi jangka panjang untuk mengendalikan penyakit tersebut. Enam galur hasil persilangan antara kentang transgenik Katahdin SP951 dengan kentang Atlantic atau Granola non-transgenik telah dipilih berdasarkan karakter agronomis dan uji ketahanan terhadap penyakit hawar daun. Penelitian ini bertujuan untuk menganalisis jumlah sisipan dan stabilitas gen* RB *pada genom galur kentang transgenik. Penelitian dilakukan melalui tahapan ekstraksi DNA tanaman diikuti dengan analisis* shouthern blot *dan* polymerase chain reaction *(PCR). Analisis* shouthern blot *digunakan untuk mendeteksi jumlah sisipan yang berintegrasi pada genom tanaman transgenik, sedangkan analisis PCR untuk mendeteksi stabilitas gen* RB *dari generasi ke generasi. Hasil penelitian menunjukkan bahwa progeni dari persilangan Atlantic dan transgenik Katahdin SP951 (galur No. 20 dan 27) dan antara Granola dan transgenik Katahdin SP951 (galur No. 69) mengandung satu sisipan gen* RB *berdasarkan analisis* southern blot *menggunakan* probe *fragmen* npt*II. Hasil ini sama dengan jumlah sisipan gen* RB *yang ada pada tanaman tetua Katahdin transgenik SP951. Keberadaan gen* RB *pada galur transgenik empat generasi yang berbeda* G_0, G_1, G_2, *dan G3 menunjukkan stabilitas integrasi gen* RB *pada genom kentang. Jumlah sisipan tunggal gen* RB *akan mengurangi terjadinya pembungkaman ekspresi gen. Analisis stabilitas gen* RB *dapat menentukan gen tersebut masih stabil keberadaannya pada genom tanaman kentang setelah beberapa generasi.*

[***Kata kunci*:** Kentang transgenik, stabilitas genetik, *Phytophthora infestans*, gen *RB*]

INTRODUCTION

Potato (*Solanum tuberosum* L.) is a member of Solanaceae family which is economically important and mostly consumed in the world along with wheat and rice. The global production of potatoes approached 330 megatons in 2009, with Asia and Europe represent the regions with the largest areas of potato production (FAOSTAT 2011). The demand for potato in big cities in Indonesia is slightly increasing, although annual per capita potato consumption in Indonesia is only around 1.35 kg, which is still relatively low. The last estimation predicted that per capita potatoes consumption had increased in about 4.7 kg in 2012 (Helgi Analytics 2014). In Java, for

example, an annual per capita consumption ranged between 0 and 50 kg depending on whether or not a person lives in a potato-producing area, as well as socioeconomic status and ethnic background.

Potato late blight caused by *Phytophthora infestans* is a devastating disease on potato. In the United States and other developed countries, management of late blight relies mostly on frequent application of fungicides because most cultivars of potato are highly susceptible. Controlling by frequent pesticide spraying resulted in a high cost and negative impact on the environment (Struik and Wiersema 1999).

Developing potato cultivars with high levels of broad spectrum resistance is a key long-term management strategy for combating this disease. An alternative strategy is the introduction of a single gene through stable transformation. The advantage of this procedure is that a highly desirable trait, conferred by one or a few genes, can be added with little chance of drastically altering the recipient plant's genetic structure or physiology under normal growth conditions. With the addition of a single resistance (*R*) gene, the resulted plant gains the ability to defend itself against a normally virulent pathogen, thus reducing the need for application of pesticides (Dangl and Jones 2001).

The development of transgenic potato Katahdin event SP951 resistant to potato late blight disease was conducted by scientists from the University of Wisconsin, Madison, USA. Katahdin SP951 was transformed using *Agrobacterium tumefaciens* LBA4044-mediated transformation method using *RB* gene which presents in plasmid pCL04541 (Ziegelhoffer *et al*. 1999; Song *et al*. 2003). Katahdin SP951 is a Katahdin potato cultivar containing *RB* gene conferring resistance to late blight disease (Halterman *et al*. 2008). *RB* gene (Song *et al*. 2003) or *Rpi-blb1* gene (van der Vossen *et al*. 2003) is a resistance gene from *Solanum bulbocastanum* that is a potentially useful control method for potato late blight and has a broad-spectrum resistance to multiple virulence factors of *P. infestans* (Kuhl *et al*. 2007). *RB* gene showed the resistance in Toluca, Mexico under intense disease pressure (Helgeson *et al*. 1998). Song *et al*. (2003) and the teams reported that five lines of 14 transformed lines were highly resistant and nine lines were moderately resistant. Transgenic Katahdin plant (event SP922) containing *RB* gene has also shown resistance to all tested isolates, including a ''super race'' in the United Stated that can overcome all 11 known *R* genes in potato (Samen *et al*. 2003).

In 2006-2007, transgenic Katahdin SP951 was tested for resistance against *P. infestans* of Indonesian isolates conducted in the confined field trial (CFT) of the Indonesian Vegetables Research Institute (IVEGRI). The results showed that Katahdin SP951 demonstrated higher tolerance to *P. infestans* of Indonesian isolates compared to non-transgenic Atlantic, Granola and Katahdin. After the resistance test, transgenic Katahdin SP951 was crossed with Indonesian local varieties, Atlantic and Granola. The crosses were carried out in the greenhouse equipped with air-conditioning and artificial light systems, in the biosafety containment facility (BCF) of the Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD).

The Atlantic and Granola hybrids were field tested in several locations (Lembang, Pangalengan, Banjarnegara and Garut) at CFTs. Regarding to the President Decree Number 21 Year 2005 Article 14 Paragraph (1) about Biosafety of Genetically Modified (GM) Product, the assessment of GM product should be done before cultivar release and commercialization. Biosafety assessments of GM products must include the stability of gene integration, substantial equivalence, unintended effects of non-target organisms, and gene flow analysis (Conner 1994; Schauzu 2000). Agronomic performance and resistance responses to *P. infestans* were investigated to select the best hybrid possibilities. The presence of *RB* resistance gene in the hybrids was confirmed using copy number and stability analysis through southern blot and PCR techniques.

The study aimed to analyze the number of insertions and the stability of *RB* resistance gene in transgenic potato lines as one of the required components of the biological safety assessment of genetically modified crops before released and commercialized.

MATERIALS AND METHODS

Plant Materials for Clonal Stability Study

Six selected clones of *RB* gene-potato tolerant to late blight were obtained from generation 0 (G_0) from CFT Lembang (2012), G1 from CFT Pangalengan (2013), G_2 from CFT Garut (2012) and G_3 from ICABIOGRAD's BCF Bogor. The negative controls were non-transgenic Atlantic, Granola and Katahdin, while the positive control was transgenic Katahdin SP951. Planting of transgenic potato and the positive and negative controls in the CFT were conducted in experimental plots using optimal procedures of potato cultivation techniques. Six selected potato clones and the control tubers were prepared and grown in

CFT after passing their dormancy treatment (3 months after the tubers harvested). The leaf samples of transgenic and control plants were taken from 3 to 4-week old plants in the CFT. While the leaf samples of plants from the greenhouse were taken from potato plants grown in pots with a mixture media of soil : rice husk : manure in the ratio of 1: 2: 2.

Genomic DNA Extraction

Genomic DNA was isolated according to Fulton *et al*. (1995). Young potato leaves of about 0.05-0.1 g (2.0-2.5 cm in diameter) were placed in a 1.5 ml micro-centrifuge tube and then incubated on ice. Leaf tissues were lyophilized into liquid nitrogen and ground to a fine powder in a 1.5 ml microcentrifuge tube with a pellet pestle. About 600 µl extraction buffer (EB) was added and mixed for 40-60 seconds until thoroughly mixed. The solution was then centrifuged for 6,500-7,000 g-force for 15 minutes at room temperature and the supernatant was discarded. The pellet was added with 250 µl EB and mixed for about 40-60 seconds. To remove the RNA, 5 µl RNase (pancreatic RNAse A) 10 mg ml^{-1} was added to the solution. A 600 µl lysis buffer (LB) enriched with 60 µl 5% sarkosyl was added into the solution. The solution was then mixed by inverting the tube for 20-40 times followed by incubation at 65°C for 15 minutes. A total of 500 µl of chloroform : isoamyl alcohol (24:1, v/v) was then added and the samples were vortex for 40-60 seconds to mix the contents. The solution was centrifuged at 6,500-7,000 g-force for 5-10 minutes at room temperature. The super-natant was then transferred into a new clean 1.5 ml microcentrifuge tube. DNA precipitation was done using ice cold isopropanol (-20°C) and DNA pellet was washed with 70% alcohol.

Copy Number Analysis

Copy number analysis of *RB* gene was performed according to the methods of Sambrook and Russell (2001) and Kramer *et al*. (2009). Approximately 20 µg of DNA from each line was digested with *Hin*dIII which did not cut *npt*II gene. Electrophoresis was carried out after the restriction, and DNA fragments were blotted onto a nitrocellulose membrane. The blots were probed with a 4,573-bp PCR fragment, which spans the *npt*II gene within the left border of the plasmid pCLD04541 (Fig. 1). The DNA fragment was amplified from pCLD04541 using forward primer 5'GCGGACGGCCAATAC TCAAC'3 and reverse primer 5'CCCTCATATCAACTACTACG'3. The probe was a non-radioactive labelled based on non-radioactive labelling detection following Roche guidelines. The DNA blot was probed overnight at 65°C using standard protocols. The probed membrane was then exposed onto X-ray film. Copy number data were based on the number of the *npt*II fragments on X-ray film that can be used to determine the copy number of the interested genes of each sample.

Stability Analysis

The stability analysis was performed by means of the presence of *RB* gene assayed by polymerase chain reaction (PCR) by following the method of Listanto *et al*. (2009). The size of PCR product expected was 619 bp, amplified from the N terminal end of the gene. The

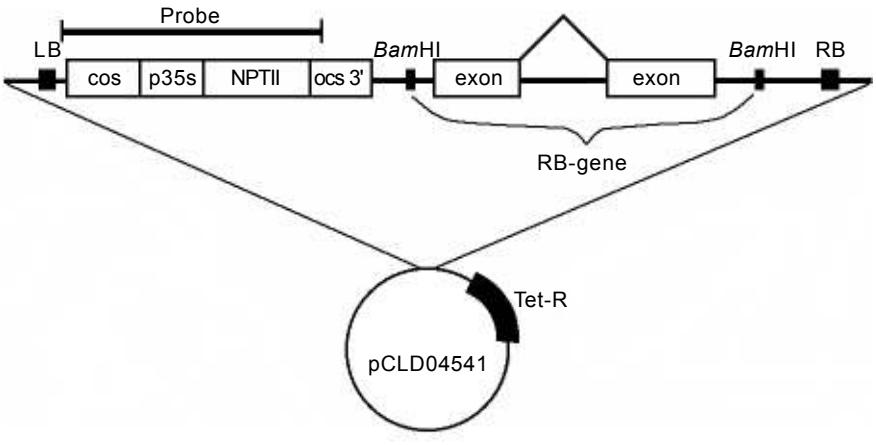

Fig. 1. Map of the *RB* gene constructs showing the 4,573-bp PCR fragment used as probes in the experiments (Kramer *et al*. 2009).

primer pairs used to amplify the fragment were F 5'-GCTCTTTGAGATTATTGCACCG AGAG-3' and R 5'-CCACCCTTTGGTGATCTGCCTT G-3'. The proof of the *RB* gene present in the potato lines was indicated by the presence of the expected DNA band with the fragment size of 619 bp.

RESULTS AND DISCUSSION

RB Gene Copy Number Analysis in Transgenic Potato Lines

Southern blot analysis showed that a single hybridization band was obtained from each of the progenies tested (Fig. 2a). The tested transgenic lines were obtained from crossing between Atlantic and transgenic Katahdin SP951 (lines No. 20 and 27) and between Granola and transgenic Katahdin SP951 (line No. 69). The copy number of *RB* genes was also observed in the transgenic Katahdin SP951. This is as expected as the transgenic Katahdin SP951 contains only one copy of *RB* gene, according to the probing of *npt*II fragment to the *Eco*RI- or *Hind*III-restricted transgenic plant genome (Song *et al*. 2003; Kramer *et al*. 2009) (Fig. 2b). Thus, a single hybridization band would indicate that the transgenic line contains a single *RB* gene insertion (one copy number). Therefore, by referring to the *npt*II fragment, the *RB* gene copy number was the same between the parental transgenic Katahdin SP951 and its crossing products. Kramer *et al*. (2009) also reported that *RB* copy

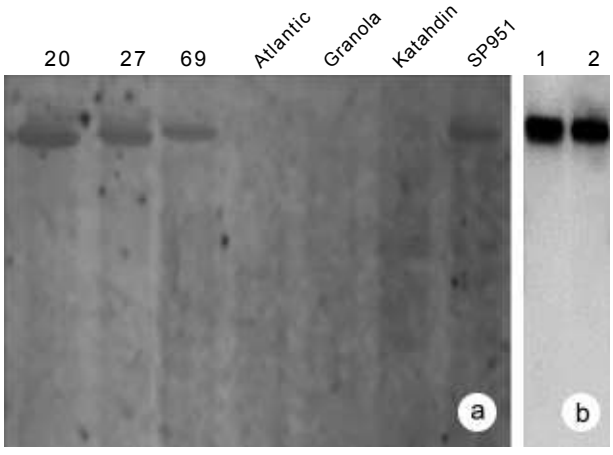

Fig. 2. Copy number analysis of *RB* gene in transgenic potato lines (a). Lanes 20, 27 and 69 were transgenic clones, followed by non-transgenic Atlantic, non-transgenic Granola, non-transgenic Katahdin and transgenic Katahdin SP951. (b) Transgenic Katahdin SP951 restricted with (1) *Eco*RI and (2) *Hind*III (Kramer *et al*. 2009). Lines No. 20, 27 and 69 demonstrated a single copy of *RB* gene, the same as that of the transgenic Katahdin SP951.

numbers of transgenic potato lines ranged from one to four copy numbers. There were significant differences in disease development between those lines. The results showed that the late blight resistance level in transgenic lines demonstrated a strong correlation with the copy number of *RB* gene. The potato lines having more copy number of *RB* gene showed a strong resistance level to late blight disease (Halterman *et al*. 2008).

Bradeen *et al*. (2009) reported the correlation of *RB* copy number transcript levels and disease resistance. They reported that disease resistance enhanced as RN copy numbers and transcript levels increased. Transgenic potato lines with 15 copies of *RB* gene maintained high *RB* transcript levels and were ranked among the most resistant among 57 potato lines tested. Consistent with this finding, several scientists working in a range of plant species have previously noted the direct correlation between gene copy number and transcript accumulation (Stockhaus *et al*. 1987; Hobbs *et al*. 1993; Ku *et al*. 1999; Schubert *et al*. 2004). Bradeen *et al*. (2009) found that in ultra-high copy number lines containing *RB* gene, innate RNA silencing has not been triggered. Schubert *et al*. (2004) reported that RNA silencing of genes only occurs when transcript levels pass a gene-specific threshold, achieved in a study by the introduction of more gene copies, by using the strong cauliflower mosaic virus 35S promoter. However, beyond a certain number of gene copies, RNA silencing was evident. But in the *RB* gene case, the gene is under the control of its native, endogenous promoter. Shen *et al*. (2002) studies were unable to detect transcript of the lettuce *R* gene *Dm3* in Northern hybridizations of total RNA and concluded that this gene, too, is transcribed at low levels. Thus, transcripts of *R* genes in general may accumulate in the plant cell at relatively low levels. In this transgenic potato containing *RB* gene, *RB* gene has low levels of *R* gene transcript accumulation mediated by endogenous promoters so that they do not fully engage RNA silencing mechanisms, even in plants containing ultra-high gene copy numbers.

Stability Analysis of *RB* Gene Integration Across Potato Transgenic Generations

Generation 0 (G_0), CFT Lembang

PCR analysis of six selected lines (No. 20, 27, 62, 65, 66 and 69) from the initial generation of G_0 lines together with all the controls (positive control of

Katahdin SP951 and negative controls of non-transgenic Katahdin, Atlantic and Granola) showed the presence of the 619 bp DNA band in all six lines and the positive control, which indicated the presence of *RB* gene (Fig. 3). As expected, the negative controls did not produce such a band.

Generation 1 (G1), CFT Pangalengan

The PCR analysis of the first generation of transgenic lines (G_1) obtained from Pangalengan field trial demonstrated the same results as that of the G_0. The six lines and the positive control (Katahdin SP951) produced PCR fragments of 619 bp which indicated the presence of *RB* gene, while the negative controls (non-transgenic Katahdin, Atlantic and Granola and blank/non-template) did not produce the expected PCR fragments (Fig. 4).

Generation 2 (G2), CFT Garut

The PCR results of the second generation (G2) obtained from Garut field trial demonstrated the same results as those of the previous two generations (G_0 and G_1). The six transgenic lines (No. 20, 27, 62, 65, 66 and 69) together with the positive control (Katahdin SP951) resulted PCR fragments of 619 bp. This indicated that the *RB* gene was present in the tested lines. The negative controls (non-transgenic Katahdin, Atlantic and Granola and blank/non-template), on the other hand did not produce any PCR fragments (Fig. 5). These results also indicated that the *RB* gene was stably inherited for three generations of transgenic plants (G_0, G_1 and G_2).

Generation 3 (G_3), BSC ICABIOGRAD

The PCR amplification products of the third generation (G_3) obtained from the biosafety containment (BSC) showed the same results as those of the all other's studies done at the previous generations (G_0, G_1, G_2 and G_3). The six lines and the positive control (Katahdin SP951) produced PCR fragments of 619 bp which indicated the presence of *RB* gene, while the negative controls (non-transgenic Katahdin, Atlantic and Granola and blank/non template) did not produce PCR fragments (Fig. 6).

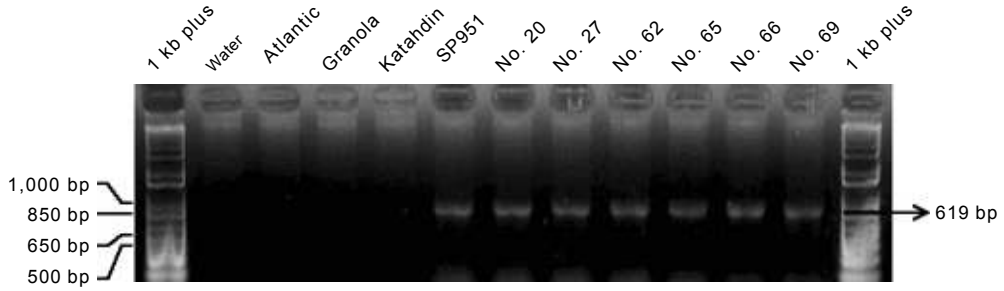

Fig. 3. PCR products of *RB* gene in selected potato lines in generation 0 (G_0), BCF Lembang, West Java. The arrow indicated the expected 619 bp PCR amplification products of *RB* gene.

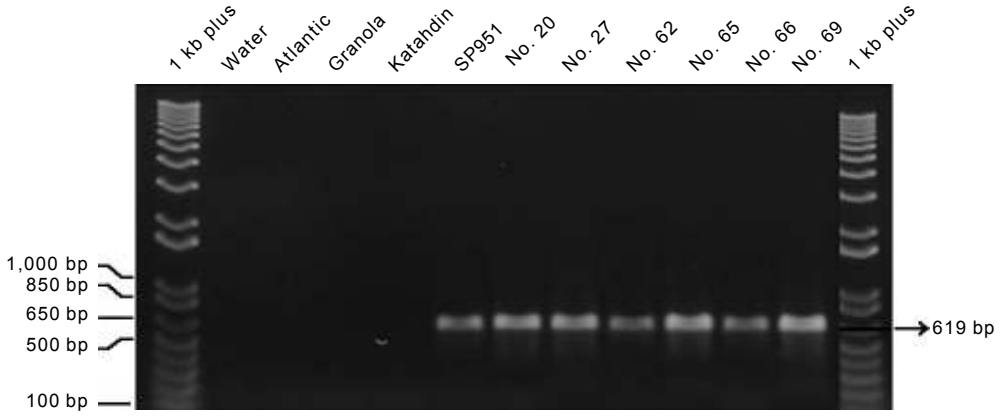

Fig. 4. PCR products of *RB* gene in selected potato lines in generation 1 (G_1), BCF Pangalengan, West Java. The arrow indicated the expected 619 bp PCR amplification products of *RB* gene.

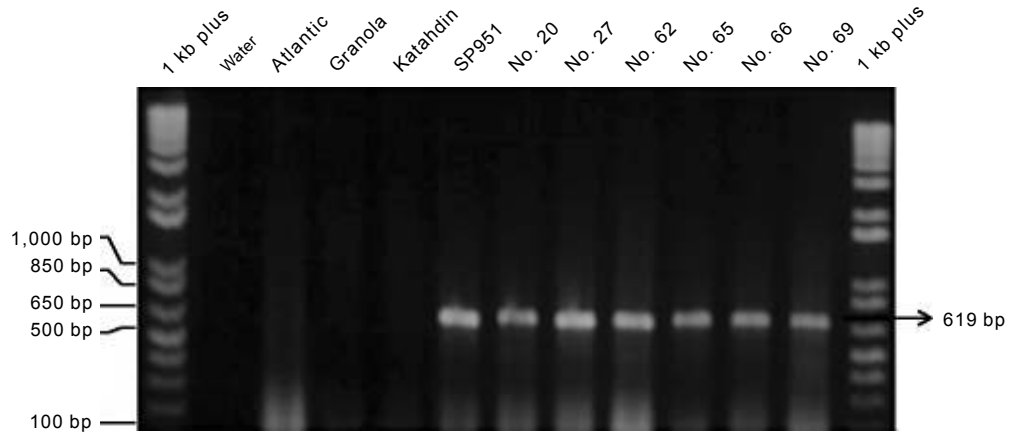

Fig. 5. PCR products of *RB* gene in selected potato lines in generation 2 (G_2), BCF Garut, West Java. The arrow indicated the expected 619 bp PCR amplification products of *RB* gene.

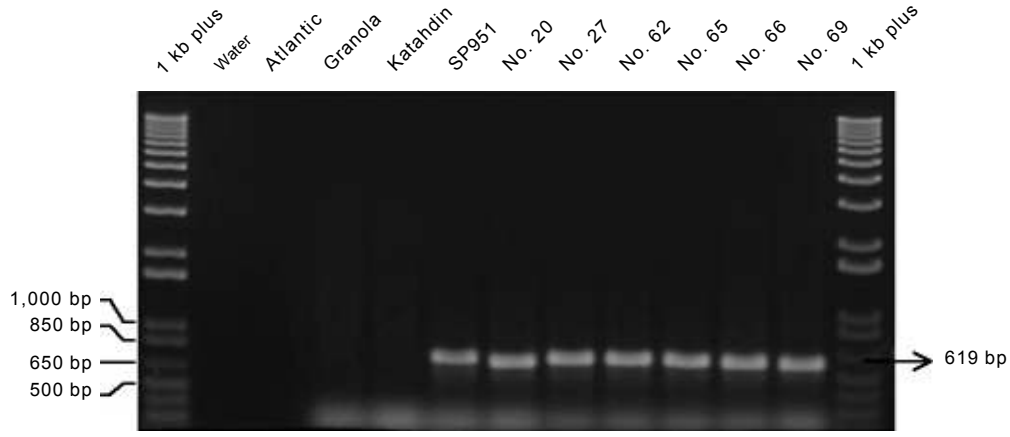

Fig. 6. PCR products of *RB* gene in selected lines in generation 3 (G_3), BCF ICABIOGRAD, Bogor, West Java. The arrow indicated the expected 619 bp PCR amplification products of *RB* gene.

As explained above, *RB* gene of the six hybrid lines was stably inherited across four different generations of the combined events from G_0 to G_3. All of the six hybrid lines tested were stably contained *RB* gene and also tolerant to *P. infestans* based on field studies at several different locations. These included CFT Lembang (Ambarwati *et al*. 2012), CFT Garut, CFT Pangalengan and CFT Banjarnegara (unpublished). All results showed that the transgenic plants tested demonstrated resistance phenotypes to *P. infestans* at any generations of the transgenic lines. An example of the performance of the transgenic plants containing *RB* gene is shown in Figure 7. This picture demonstrated that non-transgenic potatoes (Atlantic, Granola and Katahdin non-transgenic) have been damaged after infection by *P. infestans*. Whereas, transgenic lines No. 27 and 62 having the same age as the non-transgenic lines showed resistance to the pathogen; they demonstrated very similar resistance phenotypes as that of the transgenic parent (transgenic Katahdin SP951).

CONCLUSION

The progenies obtained from crossing between Atlantic and transgenic Katahdin SP951 (clones No. 20 and 27) and between Granola and transgenic Katahdin SP951 (clone No. 69) contained one copy number based on southern blot studies by probing the *npt*II fragment to the *Hin*dIII-restricted transgenic potato plants. PCR analysis of the *RB* gene in four sequential generations (G_0, G_1, G_2 and G_3) showed the stable inheritance of the *RB* gene in the genome of potato transgenic lines as indicated by the consistent appearance of the 619 bp PCR products of

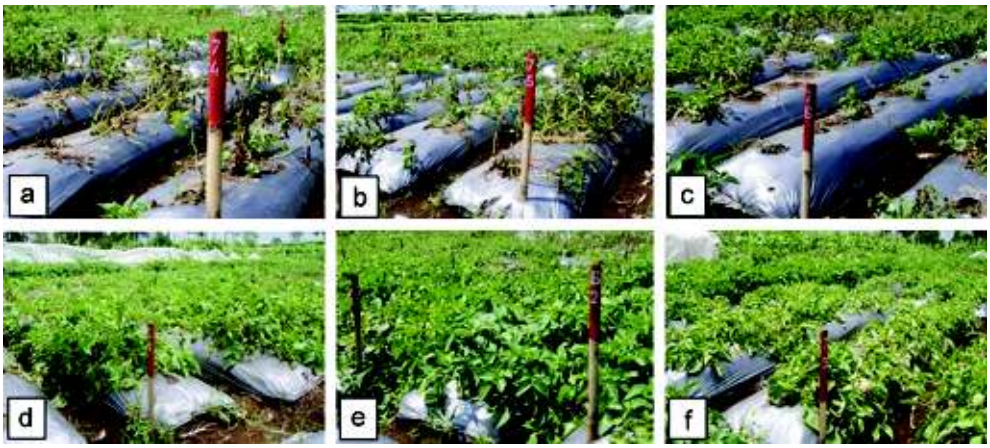

Fig. 7. Resistance performance of transgenic potato lines containing *RB* gene. a, b and c were the susceptible non-transgenic Atlantic, Granola and Katahdin, respectively. d, e and f were a hybrid of Atlantic x transgenic Katahdin SP951, a hybrid of Granola x transgenic Katahdin SP951, and transgenic Katahdin SP951, respectively.

the *RB* gene fragment in all four generations of the transgenic potato lines. The transgenic lines are good candidates for a new potato cultivar upon the completion of the required testing for releasing a new potato transgenic variety.

ACKNOWLEDGEMENT

This research was financially supported by the Agricultural Biotechnology Supporting Project (ABSP) II and government funding through ICABIOGRAD, Bogor. We sincerely thank to Prof. Muhamad Herman as a ABSPII's Country Coordinator.

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