

# Transformation using RNAi technology for developing potato lines resistance to late blight (*Phytophthora infestans*)

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**Abstract.** *Phytophthora infestans* (Mont.) de Bary causes late blight, a major disease of potato and tomato that causes production loss at about 10–100% in Indonesia. Breeding for late blight resistance in potato has been carried out in this country to minimize fungicide application. The objective of this study was to generate potato lines resistant to *P. infestans* through RNAi method. A series of studies were conducted, including confirmation of RNAi construct insertion in *Agrobacterium tumefaciens* and PCR analysis, transformation of two potato cultivars using *A. tumefaciens* carrying the RNAi fragment, in addition to selection and molecular analysis of selected plantlet using Polymerase Chain Reaction (PCR). The RNAi plasmid construct was confirmed via PCR analysis using specific primers for *35s* and *Tnos* fragment, and resulted in 500 bp and 250 bp for fragment of *35s* and *Tnos*, respectively. Transformation was performed on 733 Granola's internode explants and 569 Atlantic's internode explants. The transformation process produced 282 explants from Atlantic, while Granola did not produce any transformants. The level of transformation efficiency of Atlantic on selection medium containing hygromycin was 49.61%. Following regeneration step, the 282 selected explants produced 167 plantlets. Based on PCR reaction using specific primers for *hpt* gene, 14 plantlets were PCR positive and contained *hpt* fragment. Overall, *Agrobacterium tumefaciens*-mediated transformation on potato internode explants was successful. Therefore, the selected transformants should be further tested using bioassay for resistance to *P. infestans*.

Keywords: *Agrobacterium tumefaciens*, *Phytophthora infestans*, RNAi, potato.

## 1. Introduction

Potato (*Solanum tuberosum*) is one of the most important horticultural commodities for staple food. This commodity contains high nutritional content, including carbohydrates, proteins, minerals and amino acids, as well as some important vitamins [1]. In 2016, national potato production in Indonesia was around 1.2 million tons (MT), while national consumption was about 1.0 MT, which was higher than the production. To meet the demand, Indonesia imported around 26,000 ton potatoes. To achieve self-sufficiency in potato production and to become a potato exporter, new Indonesian variety such as Median [2] was developed and released.

The most important problem for potato production is the presence of diseases. The major disease in potatoes is late blight caused by *Phytophthora infestans* (Mont.) De Bary. This pathogen causes damages on many parts of a potato plant, such as leaves, tubers and stems. Late blight was responsible



for the European potato famine in the 19th century, which caused deaths from starvation on more than one million people in Ireland alone [3] and the migration of millions of people [4]. In Indonesia, potato production loss can reach 100% if weather conditions are conducive to the development of *P. infestans*. At present, Indonesian farmers still use several potato varieties that are susceptible to *P. infestans*, such as Granola and Atlantic. Until now, the late blight pathogen was mostly controlled using chemical pesticide. In the United States, more than 15 fungicidal sprays are applied per season, which is considered as a legal practice even though it is harmful to the environment and human health [5]. This is because more than 100 years of concerted breeding efforts across the world to develop resistant potato varieties have not been able to overcome the disease. Conventional breeding techniques that have been used since more than century ago to produce resistant lines found several obstacles, such as limited sources of resistance genes, incompatibility barrier, and insufficient expression levels of the introgressed resistance genes.

Successful development of a resistant potato to control *P. infestans* is considered as a more environmental friendly approach. New techniques have been developed to obtain resistant lines to this pathogen. Research has been initiated to introduce resistance trait from wild relatives to cultivated potato plants, identify molecular markers/QTL associated to resistance, and use genes of interest to transform varieties through cisgenesis. The *RB* gene, which is a major resistance gene from a wild relative potato *S. bulbocastanum* [6], has been mapped, isolated, cloned, and then transformed into Katahdin variety[3].

This genetically-modified potato plant resistant to late blight has been crossed with Indonesian commercial potato variety, Granola and Atlantic. The donor parent was transgenic Katahdin SP951 that carries the *RB* gene modified at the University of Wisconsin, Minnesota, USA, through ABSPII/USAID Project. Some lines among the hybrid progenies had been tested in several potato plantations in West Java and Central Java and showed resistance to late blight [7]. Transcription study of the *RB* gene indicated that in potato lines resistant to late blight the transcript levels were the highest in the foliage and tubers in an age-dependent manner. Expression of *RB* gene was the highest in young tubers and declined as the tubers aged [8].

Since the discovery of RNA interference (RNAi) technology by Romano and Macino [9], this technique is considered as an alternative technique capable of conferring enhanced resistance to fungi and pest [10]. It has been successfully applied to inhibit the growth of rice blast disease by inhibiting the growth and germination of appressoria in *in vitro* experiments [11].

The development of resistant cultivars through RNAi approach is expected to give better results, provide durable resistance, and reduce public concerns about genetically modified products. RNAi technology can be directed to degrade the pathogen's mRNA that enter the host cell or silence endogenous genes of the host cell that enable pathogen infection [12]. Compared to other methods working in protein level, such resistance mechanism in mRNA level is considered as a better approach [13]. Using this method, host-induced gene silencing of fungal genes was obtained in barley infected by powdery mildew *Blumeria graminis*, a biotrophic fungal pathogen[14]. The mechanism of pathogen control by RNAi is not dependent on the production of a foreign protein that could be allergenic or toxic in the host plants, which should make this technology more acceptable than the classic transgenic approaches for disease control [15].

RNAi technology has been considered as a promising approach for pest management, although there are some issues, such as RNAi efficiency, dsRNA degradation, and environmental risk assessments that need to be considered [16]. RNAi study has been investigated on two Kufri Indian Potato varieties (K. Khyati 1037 and K. Khyati 1129), where gene silencing method was used to silence *AVR3a* gene encoding an effector agent in *P. infestans* to infect the host plant. This investigation used siRNA and amiRNA-mediated silencing of *AVR3a* gene and produced moderate resistance against *P. infestans* [17]. The objectives of this study were to conduct *A. tumefaciens*-mediated transformation of potato internode explants and to produce potato plantlets carrying an RNAi fragment.

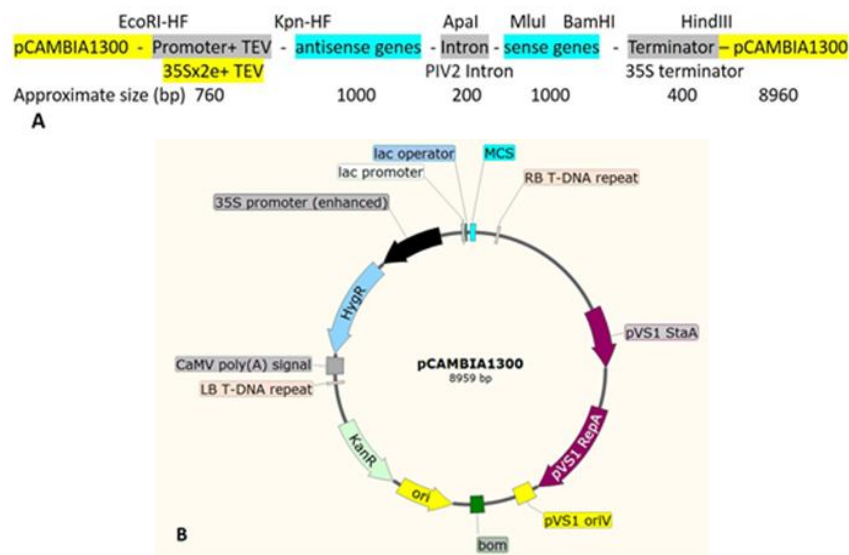
## 2. Materials and methods

### 2.1. Plant materials

Internode explants from 3–4-week-old *in vitro* cultures of Granola and Atlantic were used for transformation. The explants were grown in propagation medium (MS salts) containing  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , Myo-inositol and Phytigel Thiamine-HCl, pH 6.0.

### 2.2. Growing *A. tumefaciens* LBA4404 containing RNAi construct

A single colony of *A. tumefaciens* LBA4404 containing RNAi construct (Figure 1A; source: Venganza, Inc., Raleigh, NC, USA through ABSPII) inside a T-DNA plasmid pCambia 1300 (Figure 1B) was grown on YEM medium (mannitol [5g/l], yeast extract [0.5g/l], NaCl [0.1 g/l],  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  [0.2g/l], and pH 7.0) containing kanamycin 50 mg/l and shaken for one night at 28°C.



**Figure 1.** RNAi construct and pCambia1300 plasmid used in this study. (A) RNAi construct inside the T-DNA of pCambia1300 plasmid. (B) The map of pCambia1300 plasmid.

### 2.3. Verification of the plasmid containing RNAi construct

A single colony of *A. tumefaciens* containing RNAi construct was grown in 3–5 ml of liquid YEP medium containing kanamycin antibiotic. The culture was incubated for two nights at room temperature on a shaker set at 200 rpm. The plasmid DNA containing RNAi construct was isolated using alkaline lysis method [18]. The plasmid DNA was used for the next stage of analysis using Polymerase Chain Reaction (PCR) with primers for *hptII* gene.

### 2.4. *Agrobacterium tumefaciens*-mediated transformation on potato internode explant

Transformation was conducted according to the method used by Ziegelhoffer et al. [19]. Pieces of stem segments measuring 0.5 mm long or 0.5 mm square of leaves were placed on a filter paper soaked in liquid MS medium. About 200 explants were immersed in a suspension of *A. tumefaciens* ( $\text{OD}_{600} = 0.6\text{--}0.8$ ) for 10–20 minutes. The explants were then transferred to callus induction medium (CIM), covered with aluminum foil and incubated at 22°C for 2–3 days in an incubator. The explants were then transferred to selection induction medium (SIM) containing timentin or carbenicillin and hygromycin. Cultures were incubated for 3 weeks at 18°C with a photoperiod setting of 16 hours. The SIM was replaced every 3 weeks until green shoots appeared. About 1–2 cm of the green shoots were transferred to rooting medium (the medium of propagation) containing hygromycin and incubated at 18°C with photoperiod set at 16 hours and allowed to form strong roots.

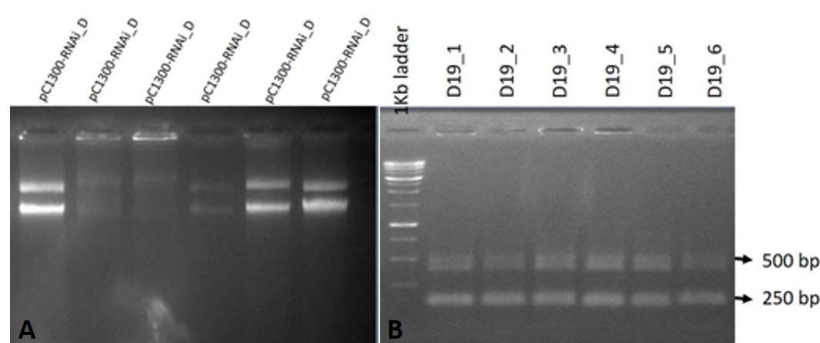
### 2.5. Molecular analysis of the putative transgenic lines

A small piece (0.5 cm<sup>2</sup>) of transformed leaf that have already rooted in the rooting medium containing antibiotic was used for plant DNA isolation based on the method used by Fulton [20] and used for PCR analysis. PCR were performed using the specific primers for *hptII* to detect the presence of the RNAi construct in the transformants following the method of Listanto [21]. Each PCR reaction contained 1× PCR buffer, dNTPs 2.5mM, 2 pmol of forward and reverse primers, 0.2 U *Taq* DNA polymerase and the DNA template. The PCR reaction was started with pre-denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 15 seconds, 61°C for 30 seconds and 72°C for 1 minute, and concluded with a temperature of 72°C for 5 minutes. The results of PCR amplification were separated in 1.5% agarose gel and visualized using Chemidoc.

## 3. Results and discussion

### 3.1. Verification of plasmids containing RNAi construct

The verification of plasmids containing RNAi construct was done through plasmid DNA isolation and PCR analysis using primers for 35s and Tnos. The results showed that the plasmid DNA that was transferred into *A. tumefaciens* LBA4404 was still intact, judged from the existence of two fragments, one of which was probably a circular DNA plasmid (Figure 2A). PCR analysis using the primers for 35s and Tnos produced two fragments of promoter 35s and terminator Tnos. The PCR products were 500 bp for 35s fragment and 250 bp for Tnos fragment (Figure 2B). These results confirmed that the RNAi construct was still inside the T-DNA of pCambia1300 plasmid. Thus, the plasmid that contained the RNAi construct was eligible to transform potato explants in order to obtain durable resistance to *P. infestans* in potato. The 35s and Tnos fragments were used to clarify the construct because these fragment are important to control DNA transcription into mRNA that would be used to silence a target gene in *P. infestans*, which is the gene suspected to control the protein expression of elicitin in *P. infestans* [22].



**Figure 2.** Verification of plasmids containing the RNAi construct using PCR method. (A) DNA plasmid band containing RNAi construct. (B) PCR amplicons of RNAi construct using 35s and Tnos primers.

### 3.2. RNAi construct transformation

The RNAi inserted into the T-DNA plasmid pCambia1300 was transferred to Granola and Atlantic explants using *Agrobacterium*. The advantage of gene transfer technique using *Agrobacterium* was that it had specific mechanisms to transfer DNA from its cell into plants using T-DNA. The molecular basis of genetic transformation by *Agrobacterium* in plant cells is DNA transfer from the bacterium followed by integration of a region of a large tumor-inducing (Ti) region into the plant nuclear genome [23]. Based on this mechanism, if a gene of interest is inserted into the T-DNA the expected gene will also be integrated into the target plant genome [24]. The *Agrobacterium*-mediated transformation

using RNAi construct into potato explants produced several plantlets that survived in the selection medium and rooting medium containing hygromycin.

**Table 1.** Number of selected explants on selection medium containing hygromycin.

Source	Number of explants	Number of surviving explants in selection medium (hygromycin 100)	Number of surviving explants shoots in rooting medium (hygromycin 50)
Atlantic (leaf)	204	1 (0.005%)	1
Granola (leaf)	66	-	-
Granola (internode)	733	-	-
Atlantic (internode)	569	282 (49.61%)	167

Hygromycin was used as a selection agent for the transformants because the pCambia1300 plasmid contains a gene (*hptII*) for resistance to hygromycin. Transformed explants which still survived in media containing hygromycin indicate that those transformants carried the *hptII* gene.

Transformation of Granola using 733 internode explants and 66 leaf explants with the RNAi construct did not produce any explants with resistance to hygromycin. However, transformation of Atlantic using 569 internode explants and 204 leaf explants produced 282 internode explants (49.61%) and one leaf explant (0.005%) resistant to hygromycin on selection medium containing 100 mg of hygromycin. The 282 selected explants produced 167 plantlets (shoots), which were transferred to rooting media containing hygromycin (50 mg). The results of RNAi construct insertion to leaf explants and internodes of Atlantic cultivars survived on selection and rooting medium containing hygromycin are shown in Table 1 and Figure 3.



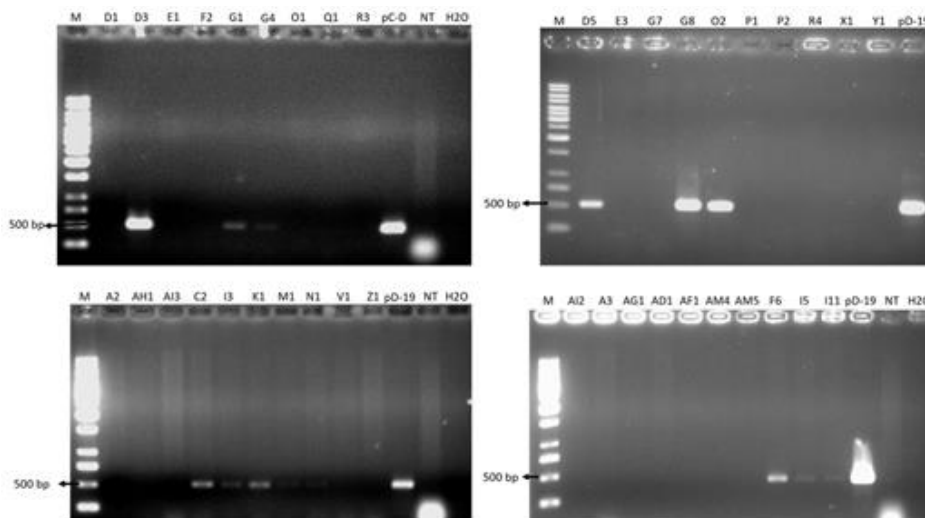
**Figure 3.** Verification of putative transformed plants containing RNAi construct. (A) Leaf and internode explants of Granola dan Atlantic on selection medium containing hygromycin. (B) Transformed plantlets on rooting medium containing hygromycin.

### 3.3. Molecular analysis of the putative transformant lines

Molecular analysis was carried out on the surviving plantlets using PCR with specific primers to detect the presence of *hptII* or the RNAi construct. Thirty-nine of the 168 plantlets were used for PCR analysis using specific primers for *hptII* gene and 14 plantlets were PCR positive. Transformed plantlets that had DNA fragment of *hptII* gene are expected to survive on medium containing hygromycin. The presence of *hptII* gene inside the genome of survived transformants can be used to indicate that the plantlets also contain the RNAi construct. This assumption is based on the structure of the RNAi construct, where *hptII* gene was inserted inside the T-DNA (Figure 1), so that when the *hptII* or *hptII* gene is inserted in the plant genome then the RNAi construct should also be in it, or vice versa. According to Gelvin [23], *A. tumefaciens* has the capability to transfer intact T-DNA into plant genomes. Based on the analysis of Bartlett et al. [25], it was demonstrated that the T-DNA inserts



itself into the genome of the target plant started from the right border (RB) and terminated on left border (LB).



**Figure 4.** PCR results of transformed plantlets (Atlantic) that contained RNAi using primers for *hptII* gene. M = 1 Kb DNA ladder, pD-19 = plasmid pCambia1300 containing RNAi construct, NT = Atlantic non-transformant, H<sub>2</sub>O = water.

The results of PCR analysis showed that the 14 transformed plantlets contained the *hptII* fragment with the size of 500 bp. The existence of *hptII* gene inside the construct is at the end of T-DNA and close to the LB of pCambia1300 plasmid (Figure 1A). This result also proved that the RNAi construct was also integrated into the genome of transformed plantlets. For further analysis, an experiment should be conducted by employing advanced PCR analysis using specific primers to determine that the transformed plantlets contain the correct construction of RNAi for resistance to *P. infestans*. Whether the resistance is durable or not bioassays to *P. infestans* must be performed in greenhouses or fields.

#### 4. Conclusions

*A. tumefaciens*-mediated transformation on potato internode explants was simple and successful. The selected transformants should be further tested using bioassay for resistance to *P. infestans*. A total of 14 plantlets were PCR-positive for *hptII* fragment, indicating that RNAi construct for durable resistance to *P. infestans* was successfully integrated into the genome of transformed plantlets of Atlantic variety. Further bioassay in greenhouses and in the field should be performed on the plantlets to identify lines with durable resistance to late blight disease.

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