

Construction of *Cry1Ac* Plasmid Vector and Its Transformation into *Agrobacterium tumefaciens*

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ABSTRAK

Konstruksi Vektor Plasmid *Cry1Ac* dan Transformasinya ke Dalam *Agrobacterium tumefaciens*. Sri Koerniati dan Alifah R. Heritiera. Introduksi gen *cry* ke dalam genom padi dapat menghasilkan tanaman tahan hama penggerek batang. Sekuen DNA yang mengode gen *cry1Ac* telah disisipkan ke dalam vektor pGEM-4Z, namun konstruk ini tidak memiliki gen penyeleksi transformasi. Tujuan penelitian ini adalah mengonstruksi vektor plasmid yang mengekspresikan gen *cry1Ac* yang memiliki gen penyeleksi transformasi dan mentransformasikannya ke *Agrobacterium tumefaciens*. Bahan yang digunakan, yaitu vektor plasmid biner pAY560325, vektor pGEM4Z-*cry1Ac*, sel kompeten *Escherichia coli* strain DH5- α , dan *A. tumefaciens* strain LBA4404. Metode yang dipakai terdiri atas pemotongan DNA plasmid dengan enzim restriksi *HindIII* dan *EcoRI*, elektroforesis, pemotongan DNA (*backbone* dan *insert*) dari gel, purifikasi, dan ligasi dengan enzim *T4 DNA ligase*. Transformasi DNA, yang diligasi ke *E. coli* dengan metode *heat shock*, diikuti dengan penanaman sel pada media seleksi, kultur koloni, isolasi, dan identifikasi DNA menggunakan enzim restriksi. Rekonfirmasi DNA dilakukan dengan pemotongan menggunakan enzim restriksi dan PCR menggunakan primer spesifik gen *cry1Ac* (F3 dan R3). Hasil penelitian berupa fragmen DNA *insert* (*ubiquitin::cry1Ac*) berukuran 3,8 kb dan vektor *backbone* (pAY560325) yang setelah diligasi dan ditransformasi ke *E. coli* menghasilkan koloni. Satu dari sepuluh koloni DNA plasmid yang terbukti benar diberi nama pAY560325-*cry1Ac*. Selanjutnya, DNA plasmid ditransformasi ke *A. tumefaciens* dengan metode elektroporasi. DNA plasmid yang diisolasi dari kultur koloni *Agrobacterium* dan dipotong dengan *HindIII* dan *EcoRI* menghasilkan fragmen DNA berukuran 9,44 kb (pAY560325) dan 3,8 kb (*ubiquitin::cry1Ac*), sedangkan dengan PCR plasmid menghasilkan pita DNA berukuran 711 bp. Vektor plasmid *cry1Ac* (pAY560325-*cry1Ac*) telah berhasil dikonstruksi dan ditransformasi ke *A. tumefaciens* dan siap ditransformasikan ke genom padi.

Kata kunci: Konstruksi vektor ekspresi, gen *cry1Ac*, *Agrobacterium tumefaciens*, penggerek batang padi.

ABSTRACT

Construction of *Cry1Ac* Plasmid Vector and Its Transformation into *Agrobacterium tumefaciens*. Sri Koerniati and Alifah R. Heritiera. Introducing *cry* genes into rice genome is reported able to produce rice plant resistant to stemborer. DNA sequence encodes *cry1Ac* gene has been inserted into pGEM4Z, but this construct does not have a selectable marker gene for selection of transformed plant cells. The research aims were to construct a plasmid vector expressing a *cry1Ac* gene that has a transformation selectable gene and to transform it into *Agrobacterium tumefaciens*. Materials used were pAY560325 binary plasmid vector, pGEM4Z-*cry1Ac* vector, *Escherichia coli* strain DH5- α and *A. tumefaciens* strain LBA4404 competent cells. The methods consisted of plasmid DNA digestion using *HindIII* and *EcoRI*, electrophoresis, DNA (*backbone* and *insert*) dissection from the gel, purification, and ligation using *T4 DNA ligase*. Transformation of ligated DNA into *E. coli* by heat shock followed by cell plating onto selection medium, colony cultured, DNA isolation, and identification using restriction enzymes. Reconfirmation was done by cutting using restriction enzyme and PCR using F3 and R3, *cry1Ac* gene specific primers. Research result were DNA fragments of 3.8 kb *ubiquitin::cry1Ac* insert and pAY560325, the backbone vector, that after ligated and transformed into *E. coli* produced colonies. One of ten colonies containing plasmid DNA was evidently confirmed and named pAY560325-*cry1Ac*. Subsequently, it was transformed into *A. tumefaciens* by electrophoration method. Plasmid DNA was isolated from *Agrobacterium* that after digested with *HindIII* and *EcoRI* produced DNA fragments of 9.44 kb (pAY560325) and 3.814 kb (*ubiquitin::cry1Ac*). While by PCR, plasmid produced DNA fragment of about 711 bp. Thus, *cry1Ac* plasmid vector (pAY560325-*cry1Ac*) was successfully constructed and transformed into *A. tumefaciens* and is ready to be transformed into rice genome.

Keywords: Construction of expression vector, *cry1Ac* gene, *Agrobacterium tumefaciens*, rice stemborer.

INTRODUCTION

Rice stemborer is one of the major pests caused high yield losses of rice production in Indonesia and other countries in Asia, America, and Australia (Baehaki, 2013; Samudra, 2011; Wünn *et al.*, 1996). An

effort to reduce such losses is transferring insecticidal *cry* gene into the genome of the rice plant. That effort was made because pest control using pesticides are considered ineffective, relatively expensive, and harmful to the environment (Baehaki, 2013; Samudra, 2011; Sulistyowati, 2005). When a *cry* gene driven by promoter was inserted into the rice genome, it may change rice become resistant to this pest. Transgenic plants become resistant due to the nature of crystal protein that works as insecticide (insecticidal crystal protein) produced in the plant tissue (Nayak *et al.*, 1997; Wu *et al.*, 2002).

In nature, insecticidal *cry* genes are expressed by *Bacillus thuringiensis* (Bt), a soil bacterium, during sporulation. The gene produces a crystal protein (Bt toxin) that is toxic when hydrolyzed in the gut of insects. Many strains of *B. thuringiensis* produce more than one insecticidal crystal protein. For example, HD-1 strain of *B. thuringiensis* subsp. *kurstaki* has various *cry* genes: *cry1Aa*, *cry1Ab*, *cry1Ac*, *cryA*, dan *cry2B* (Ge *et al.*, 1989; Milne *et al.*, 1990).

All *cry1* proteins are closely related by sequence, including *cry1Aa*, *cry1Ab*, and *cry1Ac*, which have about 85% similarity in their amino acid sequences. The crystal structure of *cry1A* showed a high degree of similarity with the structures of other known *cry* proteins (*cry3A*, *cry2A*, *cry4A*, and *cry4B*) (Bravo *et al.*, 1997; Crickmore *et al.*, 1998). Each of these genes has its own insecticidal specificity, and the specificity determines the nomenclature of the *cry* proteins. For example, *cry1* protein has specific activity against pests from the order Lepidoptera (Maqbool *et al.*, 1998). Due to its high specificity, the endotoxin is safe to use and is not toxic to nontarget insects, birds, and mammals (Tu *et al.*, 2000). Lack of toxicity towards other organism cells is particularly important when a protein to be considered as a suitable candidate for the pest control by its expression in transgenic crops or for a new insecticidal design.

In this study, a *cry1Ac* gene was chosen to construct into plasmid vector, because *cry1Ac* protein has a broader attack effectiveness compared with *cry1C* and *cry2A* proteins. Nevertheless, these three genes have similar toxicity to yellow stemborer, a major target of stemborer (Lee *et al.*, 1997). Moreover, rice transgenic expressing *cry1Ac* gene showed high toxicity to striped stemborer and yellow stemborer (Cheng *et al.*, 1998; Nayak *et al.*, 1997). The objectives of this study were to construct a plasmid vector expressing of *cry1Ac* gene that has a selectable gene for transformation and to transform it into *A. tumefaciens*. Experiments carried out, results

obtained, and further plans were presented and discussed.

MATERIALS AND METHODS

The experiments were carried out in the Molecular Biology Laboratory of the Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD), Bogor, from June 2011 to January 2012.

Bacterial Strains and Plasmids

pGEM-4Z (Promega), a vector containing *cry1Ac* gene, was provided by Dr. Illimar Altossaar, from University of Ottawa, Canada (Ida Hanarida, personal communication, 2005), while pAY560325, a plasmid used as a binary vector for rice genetic transformation, was provided by Dr. Pieter B.F. Owerkerk, from the Institute of Biology, Leiden University, The Netherlands (Pieter B.F. Owerkerk, personal communication, 2007). Competent cells of *Escherichia coli* strain DH5- α used for multiplication of plasmids and *A. tumefaciens* strain LBA4404 used for rice transformation were prepared using Inoue method (Inoue *et al.*, 1990).

Enzymes and Primers

Experiments were carried out using both restriction (*Hind*III, *Bam*HI, and *Eco*RI) and modification (T4 DNA ligase) enzymes. The *Hind*III, *Bam*HI, and *Eco*RI restriction enzymes were used to digest pGEM-4Z, a plasmid DNA. Beside that, to confirm the plasmid DNA, primer pairs specific for amplifying the *cry1Ac* gene (F3: 5'-CTGTACGGATAGGGTAGGTT-3' and R3: 5'-TACTTGGTGGAGAACGCATT-3') were also designed.

Preparation of Competent Cells

E. coli strain DH5- α competent cells were prepared using Inoue *et al.* (1990) method. Inoue buffer (1 L containing 10.88 g $MnCl_2 \cdot 4H_2O$, 2.2 g $CaCl_2 \cdot H_2O$, 18.65g KCl, and 20 ml of 0.5 M PIPES [pH 6.7] [Piperazine-1,2-bis{2-ethanesulfonic acid}]) was prepared. Subsequently, 100 μ l glycerol stocks of *E. coli* strain DH5- α was cultured in 5 ml LB medium, agitated at 250 rpm for 16 hours at 37°C. Two milliliters of starter culture was transferred into 50 ml LB medium. This culture was agitated at 250 rpm for 6 hours at 37°C. Cells were harvested at an OD_{600} of 0.5 by centrifugation at 4,000 rpm for 10 minutes at 4°C. A pellet of cells was then resuspended using ice-cold Inoue buffer and recentrifuged. Lastly, the pellet was resuspended, divided into small volume (aliquots),

snap-frozen using liquid nitrogen, and ready to use for DNA transformation using heat shock method.

Construction of Expression Vector and Confirmation of Results

pGEM-4Z containing DNA sequence encoding *cry1Ac* that subcloned between the *Hind*III and *Bam*HI restriction sites was investigated for the *cry1Ac* orientation within the plasmid. Steps of construction were presented in Figure 1.

First, the *cry1Ac* gene driven by the ubiquitin promoter was cut off from the pGEM-4Z plasmid using *Hind*III and *Eco*RI restriction enzymes (Figure 1.1). A binary plasmid of pAY560325 was also digested with

the same restriction enzymes (Figure 1.2). Resulted fragments were run by electrophoresis in 1% agarose gel. DNA fragments of *cry1Ac* driven by the ubiquitin promoter and pAY560325 (Figure 1.3) were then dissected from the gel and purified using gel extraction kit (Qiagen). One microliter of the purified DNA was then checked by electrophoresis using 1% agarose gel. The gel was stained by ethidium bromide and visualized using chemidoc (Biorad). After confirmation, these two DNA fragments were used in ligation experiment using T4 DNA enzyme to produce a recombinant plasmid of pAY560325 containing *cry1Ac* gene driven by the ubiquitin promoter (Figure 1.4).

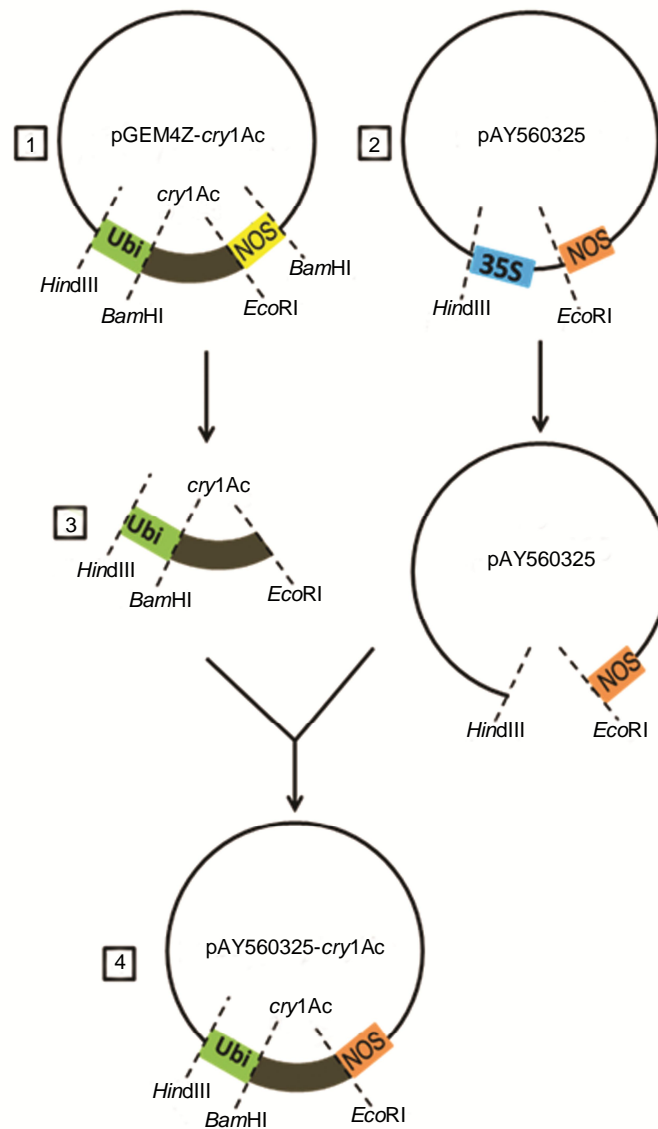


Figure 1. Steps of construction of the *cry1Ac* expression vector, namely pAY560325-*cry1Ac*.

Transformation and Confirmation of Results

To develop a recombinant plasmid, pAY560325 containing *cry1Ac* gene, the ligation reaction was then transformed into *E. coli* strain DH5- α . The transformation was done by a heat shock method. Transformed *E. coli* cells were then plated onto Luria Broth agar (LBA) medium containing 100 mg/l kanamycin, 0.1 mM/IPTG, and 40 mg/l X-gal. The culture plates were incubated at 37°C for 16 hours. Ten random colonies (white colonies) were picked and grown in liquid LB media containing 100 mg/l kanamycin, and agitated for 16 hours at 37°C. Plasmid DNAs were then isolated from the cells using the alkaline lyses method (Sambrook and Russell, 2001). The presence of *cry1Ac* gene in pAY560325-*cry1Ac* plasmid was checked by digesting DNA using combination of *Bam*HI, *Hind*III, and *Eco*RI restriction enzymes. Upon the confirmation result, the pAY560325-*cry1Ac* was then transformed into *Agrobacterium* competent cells by electroporation method. *Agrobacterium* competent cells were prepared with rather similar procedure, except using sterile water for three times washing of the pellet and 10% glycerol for the last washing and were resuspended before divided them into aliquots (Sambrook and Russell, 2001). Transformed cells were then plated onto solid YEP medium containing 100 mg/l kanamycin and 20 mg/l rifampicin. Culture plates were incubated at 28°C for 48 hours. The presence of constructed vector in the *Agrobacterium* transformed cell was checked using *Eco*RI restriction enzyme. DNA was isolated from culture, as mentioned above. PCR technique using F3 and R3 specific primers for *cry1Ac* gene was also performed to reconfirm the presence of pAY560325-*cry1Ac* within the *Agrobacterium* cell. All digestion and PCR reactions were checked by electrophoresis in 1% agarose using 1 \times TAE buffer (1M Tris-HCl and EDTA). *Agrobacterium* culture containing the correct plasmid construct was then kept in a glycerol stock and stored at -80°C.

RESULTS AND DISCUSSION

Preparation of Competent Cells

To check whether cells of *E. coli* strain DH5- α prepared were competent or not, we did transformation of plasmids used for cloning. Results showed that we had good competent cell for our experiments.

Construction of Plasmid Vector and Confirmation of Results

A schematic of cloning steps carried out to construct the pAY560325-*cry1Ac* plasmid vector was

presented above (Figure 1). A binary vector of pAY560325 was chosen as a backbone for a plasmid vector of *cry1Ac* expression, since it has suitable restriction sites at its multiple cloning site for inserting a DNA fragment containing *cry1Ac* gene driven by the ubiquitin promoter. It also has *hptII* gene resistant to hygromycin driven by the 35S promoter for selection. From the first step, in which the DNA fragment containing *cry1Ac* gene driven by the ubiquitin promoter was cut off from pGEM4Z plasmid using *Hind*III and *Eco*RI (Figure 1.1), two fragments of 3.8 kb (the DNA fragment containing *cry1Ac* gene driven by the ubiquitin promoter) and 3.1 kb were produced (Figure 2A) and those were as expected (illustration produced by VNTI Advance 11 program) (Figure 3A). pAY560325 plasmid that was digested using the same restriction enzymes produced two DNA fragments of 9.45 kb which was the backbone and 483 bp which was the 35S promoter (Figure 2B), and these results were correct (as expected that produced by VNTI Advance 11 program) (Figure 3B).

Both DNA fragments (*cry1Ac* gene driven by the ubiquitin promoter and pAY560325 plasmid) were dissected out from gels and purified using gel extraction kit (Qiagen). One microlitre of purified DNAs was then checked by electrophoresis using 1% agarose gel. Results showed that those two DNAs were correct in size and had enough concentration and quality for ligation reaction (Figure 2C). The ligation reaction was then transformed into *E. coli* competent cell by heat shock method and it produced blue and white colonies. Ten white colonies were cultured in LB medium containing 100 mg/l kanamycin and an alkalis lyses method was performed to isolate their DNAs (Figure 4, left). DNA was then digested using *Hind*III and *Eco*RI restriction enzymes for confirmation (Figure 4, right). Results showed that only one of ten plasmids (number 8) was evidently having the correct size. It produced 9.45 kb and 3.8 kb DNA fragments which were the pAY560325 (the backbone) and the *cry1Ac* gene fused to the ubiquitin promoter (the insert), respectively (Figure 1.4 and Figure 4).

A further confirmation was carried out by digesting the plasmid DNA no. 8 with several combinations of restriction enzymes. The size of DNA fragments produced from digestions was compared to what were expected (produced using VNTI Advance 11 program) (Figure 5).

Four combinations of restriction enzymes were made for confirming the plasmid no. 8. Firstly, the plasmid was digested with *Hind*III and *Bam*HI restriction enzymes. It produced two DNA fragments

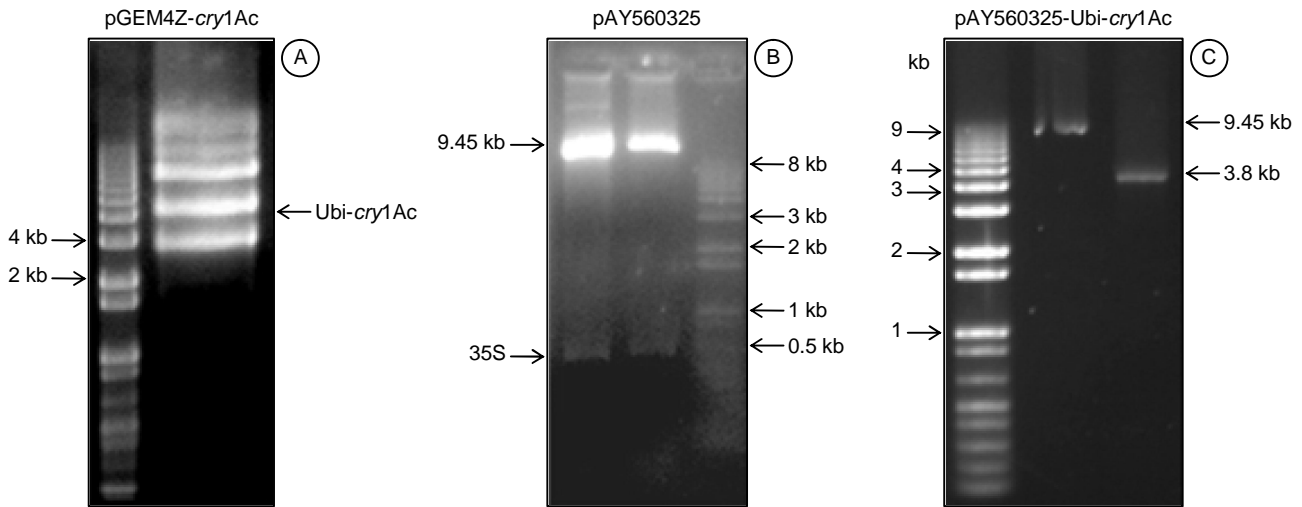


Figure 2. Electrophoresis of digestion reactions and DNA fragments used in ligation reactions. A = pGEM4Z-*cry1Ac*, B = pAY560325, C = purified DNA (of ubiquitin::*cry1Ac* and pAY560325 without 35S promoter).

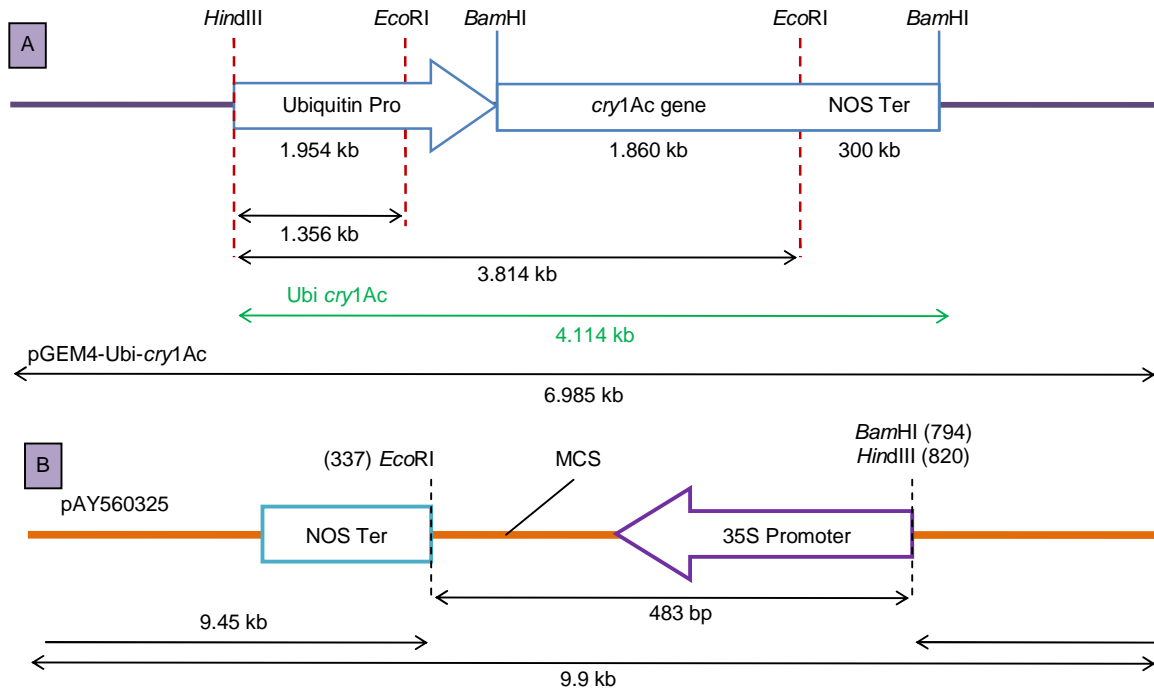


Figure 3. Illustrations of pGEM4-Ubi-*cry1Ac* (A) and pAY560325 (B) plasmid DNAs when both were digested by *Hind*III and *Eco*RI restriction enzymes (produced using VNTI Advance 11 program).

of about 1.954 kb (a part of the ubiquitin promoter) and 11.3 kb (the most rest of pAY560325-*cry1Ac*) (Figure 6) and these were as expected (Figure 5).

Based on these results, it can be confirmed that the experiment produced the correct plasmid vector, and it was named pAY560325-*cry1Ac*. Thus, it can be continued to transform the plasmid into *Agrobacterium* competent cells using an electroporation method. Cells were grown on YEP

solid medium containing 100 mg/l kanamycin and 20 mg/l rifampicin at 28°C for 48 hours. Five colonies were cultured in YEP liquid medium containing similar antibiotics and plasmid DNA was isolated from those cultures. Reconfirmations of plasmid DNA were carried out by digestion using *Eco*RI and by PCR using F3 and R3 primers. Digestion reaction results showed that two DNA fragments of about 2.46 kb (a part of the ubiquitin promoter and *cry1Ac* gene) and 10.790 kb

(the most rest of pAY560325-*cry1Ac*) were produced (Figure 7). These results were similar to DNA that was

isolated from *E. coli* and was digested by *EcoRI* (Figure 8 line 3).

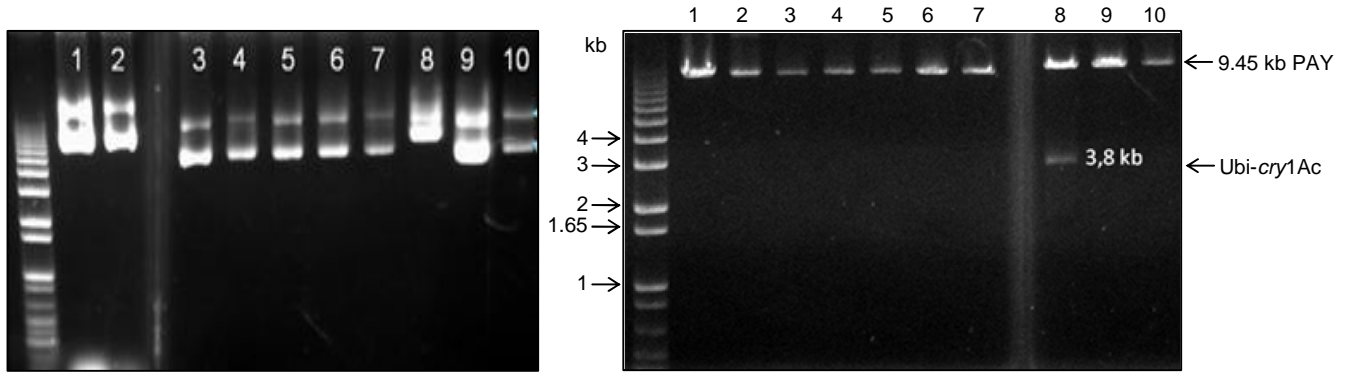


Figure 4. Electrophoresis of recombinant plasmid DNA (pAY560325-*cry1Ac*), before (left) and after digested with *HindIII* and *BamHI* (right). 1–10 = sampled colonies of *E. coli* (were expected to contain the recombinant plasmid).

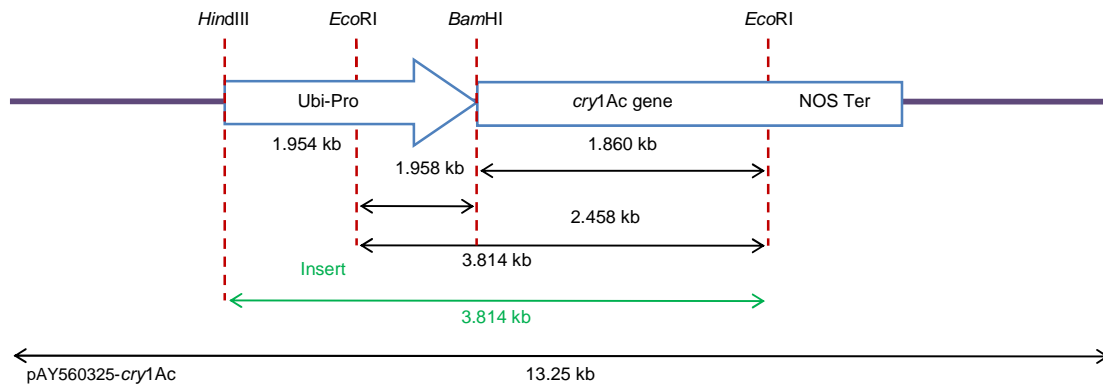


Figure 5. Diagram indicating size of DNA fragments expected to produce after double digestion with different combination of restriction enzymes (*HindIII*, *BamHI*, and *EcoRI*) produced using VNTI Advance 11 program.

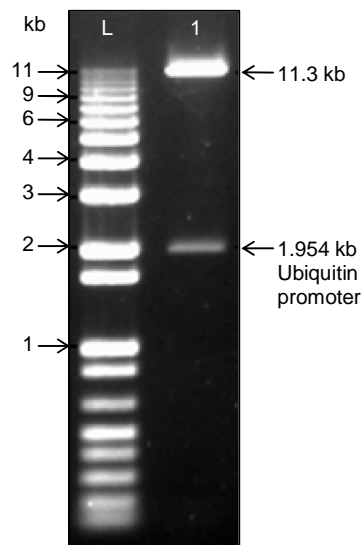


Figure 6. Electrophoresis of pAY560325-*cry1Ac* plasmid digested with restriction enzymes. L = 1 kb DNA ladder (Invitrogen) and 1 = DNA fragments produced when digested with *HindIII* and *BamHI*.

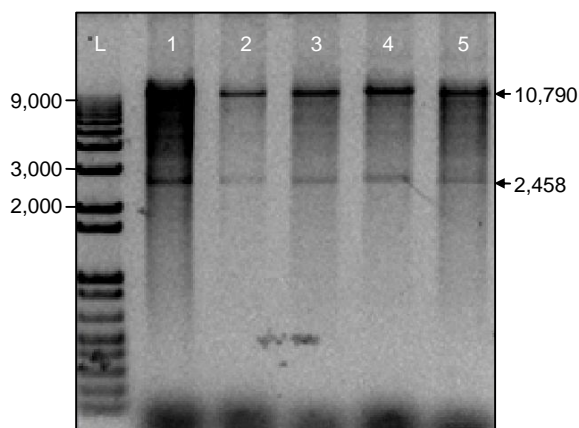


Figure 7. Electrophoresis of pAY560325-*cry1Ac* plasmid isolated from *Agrobacterium* and then digested with *EcoRI*.

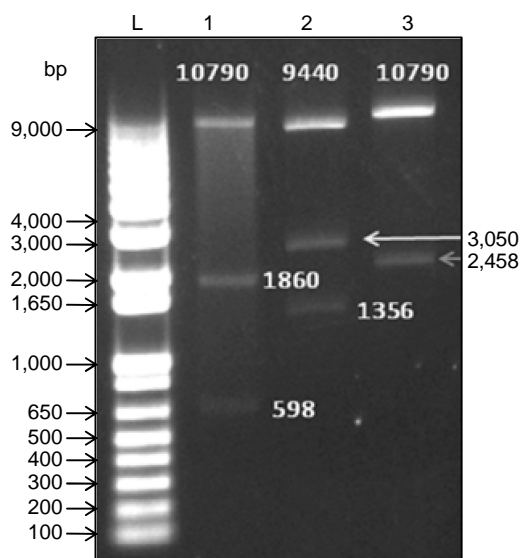


Figure 8. Electrophoresis of pAY560325-*cry1Ac* plasmid digested with restriction enzymes. L = 1 kb ladder (Invitrogen). DNA fragments produced when digested with: 1 = *Bam*HI and *Eco*RI, 2 = *Hind*III and *Eco*RI, and 3 = *Eco*RI.

Two DNA samples were used as PCR template and reaction was expected to amplify of about 711 bp of the *cry1Ac* gene (Figure 9A). PCR results showed that these two plasmids produced the expected size of DNA fragment, a position that was between 600 bp and 850 bp of 1 kb DNA ladder (Figure 9B). It means that these *Agrobacterium* cultures were proved containing the pAY560325-*cry1Ac* plasmid vector.

CONCLUSIONS

The plasmid vector of *cry1Ac* gene, named pAY560325-*cry1Ac*, was successfully constructed. The *cry1Ac* gene is under the control of the ubiquitin promoter at its 5' end and the polyadenylation sequence of NOS poly A at its 3' end. The expression vector also contains an *hptII* gene driven by the 35S

promoter that is useful for a marker for selecting transformed cells and transgenic plants generated from transformation. The pAY560325-*cry1Ac* plasmid was also successfully transformed into *A. tumefaciens* strain LBA4404 and it is currently being used in *Agrobacterium-mediated* transformation of *indica* rice varieties: Kasalath, Ciherang, and Inpari 13.

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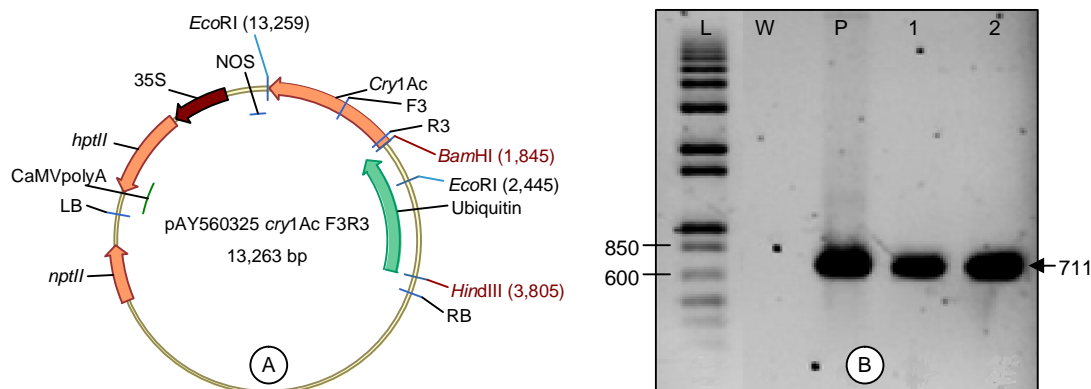


Figure 9. Diagram of recombinant pAY560325-*cry1Ac* plasmid and electrophoresis of *cry1Ac* DNA fragment produced by PCR. A = pAY560325-*cry1Ac* plasmid, T-DNA pAY560325-*cry1Ac*: position between left border (LB) and right border (RB) CaMV poly A-*nptII*-35S-NOS-*cry1Ac*-Ubiquitin (generated using VNTI 11 Advance program; Invitrogen), B = electrophoresis of DNA fragment produced by PCR using F3 and R3 primers. L = 1 kb DNA ladder (Invitrogen), W = water (negative control), P = pAY560325-*cry1Ac* plasmid (positive control), 1 and 2 = DNA isolated from *A. tumefaciens* strain LBA-4404 cultures.

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