Molecular cloning of wasabi defensin gene into plasmid containing bar gene

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Abstract

Wasabi defensin gene is responsible for soft rot disease resistance. Bar gene is responsible for herbicide resistance. In the present study, we cloned the wasabi defensin gene into plasmid containing bar gene. Hence, we obtained plasmid containing wasabi defensin gene and bar gene useful for orchids genetic transformation. Firstly, the plasmid containing bar gene was cut by HindIII restriction enzyme and the plasmid containing wasabi defensin gene was also cut by HindIII. The fragments were then ligated. The ligation product was transformed into *E. coli* competent cells and colonies were observed. Thereafter, the colonies were grown overnight in LB medium. Subsequently, the plasmids were minipreped and electrophoresis was performed. The result indicated that the ligation was occurred. The ligation product was then cut by BamH1 restriction enzyme and electroferogram showed three fragments. Subsequently, the ligation product was cut by HindIII and electroferogram showed two fragments. Thereafter, the plasmid of ligation product was transformed into *Agrobacterium tumefaciens* EHA 105 competent cells and confirmed by PCR. The result showed that the wasabi defensin and bar genes existed in plasmids cloned into *A. tumefaciens* EHA 105. Therefore, it was concluded that wasabi defensin gene has been successfully cloned into the plasmid containing bar gene.

Introduction

Phalaenopsis amabilis is one of the most important orchids grown for commercial production of cut flowers and potted plant, and is becoming an important ornamental plant in the world. The orchid free flowering with inflorescences bearing to more than 30 flowers, flowering 2 to 3 times a year and has a long-lasting vase-life making it ideal for global cut-flower markets (Pridgeon, 1992).

For further improvement of orchid species, genetic transformation is now expected to be a tool for introducing traits such as disease resistance, herbicide resistance, flower colour and shape, which has been difficult through conventional breeding techniques based on sexual crossing (Mishiba et al., 2005). One effective alternative procedure is genetic transformation either by direct delivery of genes into plant cells by particle bombardment or indirectly through the mediation of *Agrobacterium* (Belarmino and Mii, 2000). To deliver the genes into plant cells a binary vector containing desired genes must be created by molecular cloning.

Molecular cloning involves the insertion of restriction endonuclease-generated DNA fragments into a single restriction site of a cloning vector (plasmid). Individual ligated DNA molecules comprised of the vector and an additional place of DNA are then cloned by transformation into cells of a suitable host bacterium. Detection of transformants is often performed by selection for an antibiotic resistance marker carried by the plasmid (Widera et al., 1978).

In this study, two important traits such as soft rot disease resistance and herbicide resistance were cloned. Thereafter, the cloned DNA was transformed into *Agrobacterium tumefaciens* competent cells EHA 105 for subsequently used in genetic transformation of *P. amabilis* by particle bombardment and mediated by *Agrobacterium tumefaciens*.

Material and Methods

1. T-DNA and binary vector
   T-DNA region of the binary vector containing wasabi defensin gene for soft rot disease resistance is shown in figure 1. Only wasabi gene with CaMV 35S Promoter and Nos-Terminator was used in this study. Figure 2 shows binary vector or plasmid EBis-KB1 containing bar gene. In this study, wasabi defensin gene with its promoter and terminator was cloned into the plasmid EBis-KB1 containing bar gene.

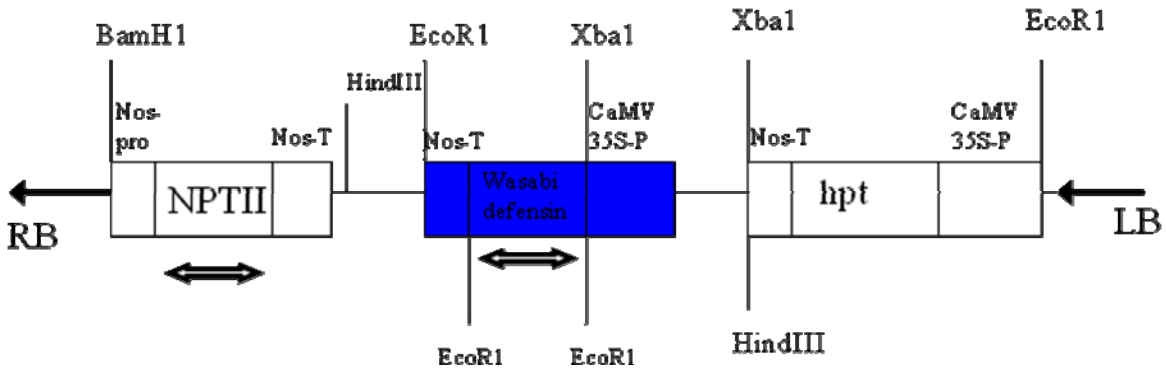


Figure 1. T-DNA region of the binary vector containing wasabi defensin gene. Wasabi defensin gene is driven by CaMV 35S promoter.

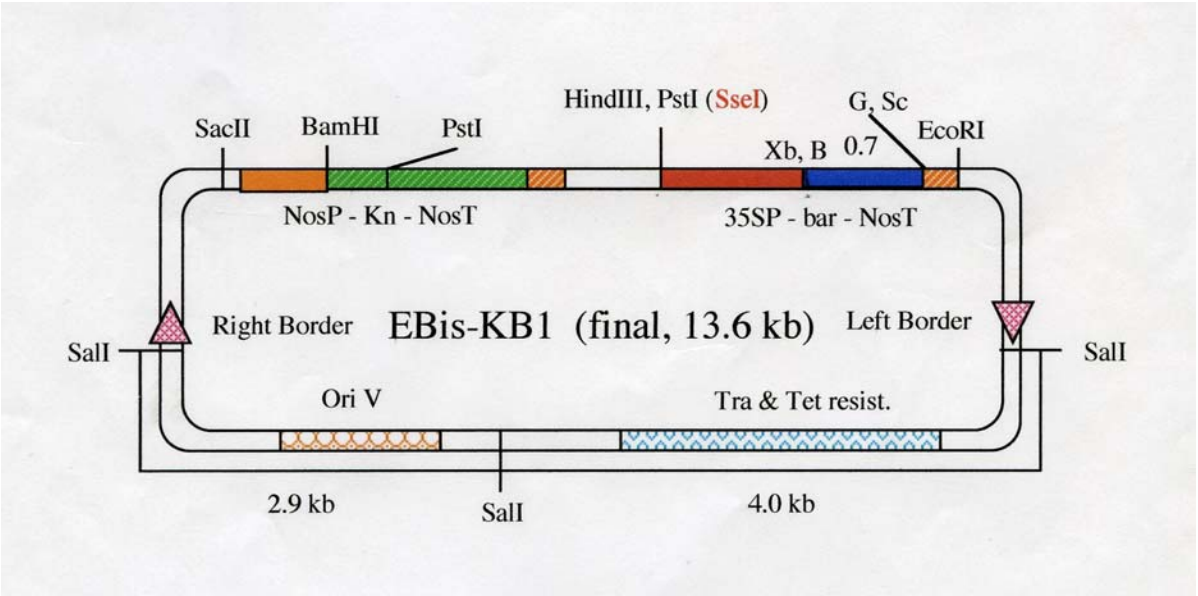


Figure 2. Binary vector EBis-KB1 containing bar gene. The bar gene is driven by CaMV 35S promoter.

2. Restriction and ligation
   Firstly, the T-DNA containing wasabi defensin gene in fig. 1 and the plasmid EBis-KB1 in fig. 2 were cut by HindIII restriction enzyme in 37^{o} C overnight. Thereafter, they were ligated in 26^{o} C for 10 min. Subsequently, the ligation product was transformed into Top 10 (Competent cells) until the colonies appeared.

3. Alkaline Lysis Plasmid Miniprep and electrophoresis
   Each one ml LB media supplemented with 30 mg/l Kanamycin and 50 mg/l Spectinomycin was inoculated with medium sized bacterial colony and incubated in 37^{o} C for overnight. Alkaline Lysis Plasmid Miniprep was then performed. Thereafter, the minipreped plasmids were electrophoresised.

4. First confirmation of ligation product by cutting with restriction enzymes
   Firstly, the plasmid of ligation product was cut by BamH1 restriction enzyme and electrophoresised to see three fragments of DNA. Secondly, the plasmid of ligation product was cut by HindIII restriction enzyme and electrophoresised to see two fragments of DNA.

5. Second confirmation of ligation product by PCR

   The plasmid of ligation product was transformed into *Agrobacterium tumefaciens* EHA 105 competent cells and minipreped. To confirm the occurrence of wasabi defensin gene, bar gene and nptII gene in plasmid of ligation product, PCR was performed. A set of primers specific to the regions of the CaMV35S promoter (F) 5'-GAT GTG ATA TCT CCA CTG AC-3' and the NOS terminator (R) 5'-CGC AAG ACC GGC AAC AGG AT-3', respectively, were used to amplify both 0.7-kb fragment of the wasabi defensin gene and 0.85-kb fragment of the bar gene simultaneously. A set of primers specific to the regions of the nptII (F) 5'-GAG GCT ATT CGG CTA TGA CTG-3'and nptII (R) 5'-ATC GGG AGC GGC GAT ACC GTA-3', were used to amplify 0.7-kb fragment of the nptII gene. PCR amplification was carried out by using the following conditions: 94^{o}C-4min 1 cycle, 94^{o}C-1min 30 cycles, 59^{o}C-1min 30 cycles, 72^{o}C-1min 30 sec 30cycles, 72^{o}C-5min 1 cycle.

Result

1. Ligation product

The electroferogram of ligation product was shown in figure 3 and 4. Figure 3 showed DNA from colony number 1 to colony no 16. Colony no. 1-6, 8,10, 12,14 and 15 showed one band. Figure 4 showed DNA from colony number 7 to colony number 24. Colony no 17-19, 21, and 24 showed one band. They indicated that T-DNA in figure 1 and binary vector in figure 2 has been ligated and showed one band of DNA.

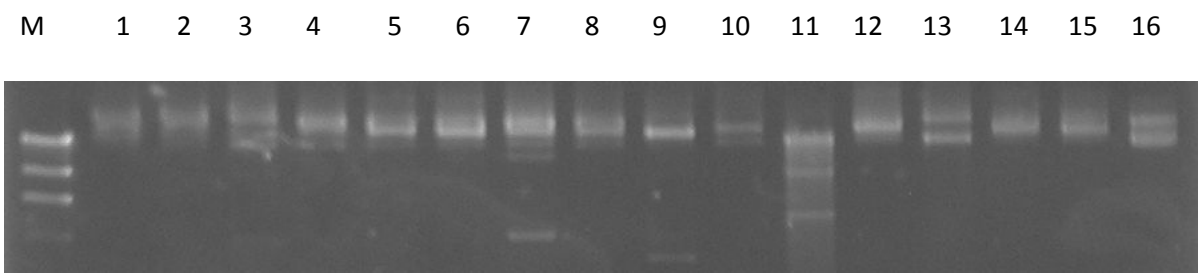


Figure 3. Electroferogram of ligation product, colony number 1-16

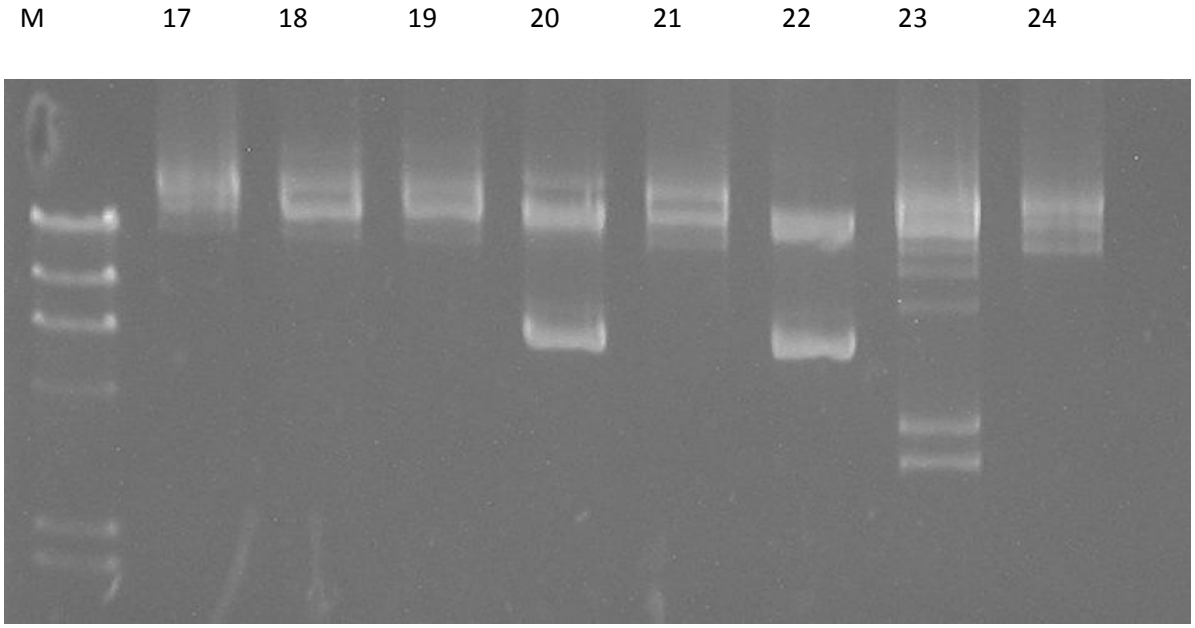


Figure 4. Electroferogram of ligation product, colony number 17-24

2. First confirmation of ligation product by cutting with BamH1 and HindIII restriction enzymes

In first confirmation, the ligation products of plasmid that showed one band in electroferogram was cut by restriction enzyme BamH1. The successful ligation products of plasmid showed three bands (marked) (Figure 5). The three bands indicated that three fragments of DNA was cut by BamH1. The positions of BamH1 were shown in figure 8. Subsequently, the ligation product of plasmid that showed one band in electroferogram was cut by restriction enzyme HindIII. The successful ligation products of plasmid showed two band (Figure 6). The two bands indicated that two fragments of DNA was cut by HindIII. The positions of HindIII were shown in figure 8.

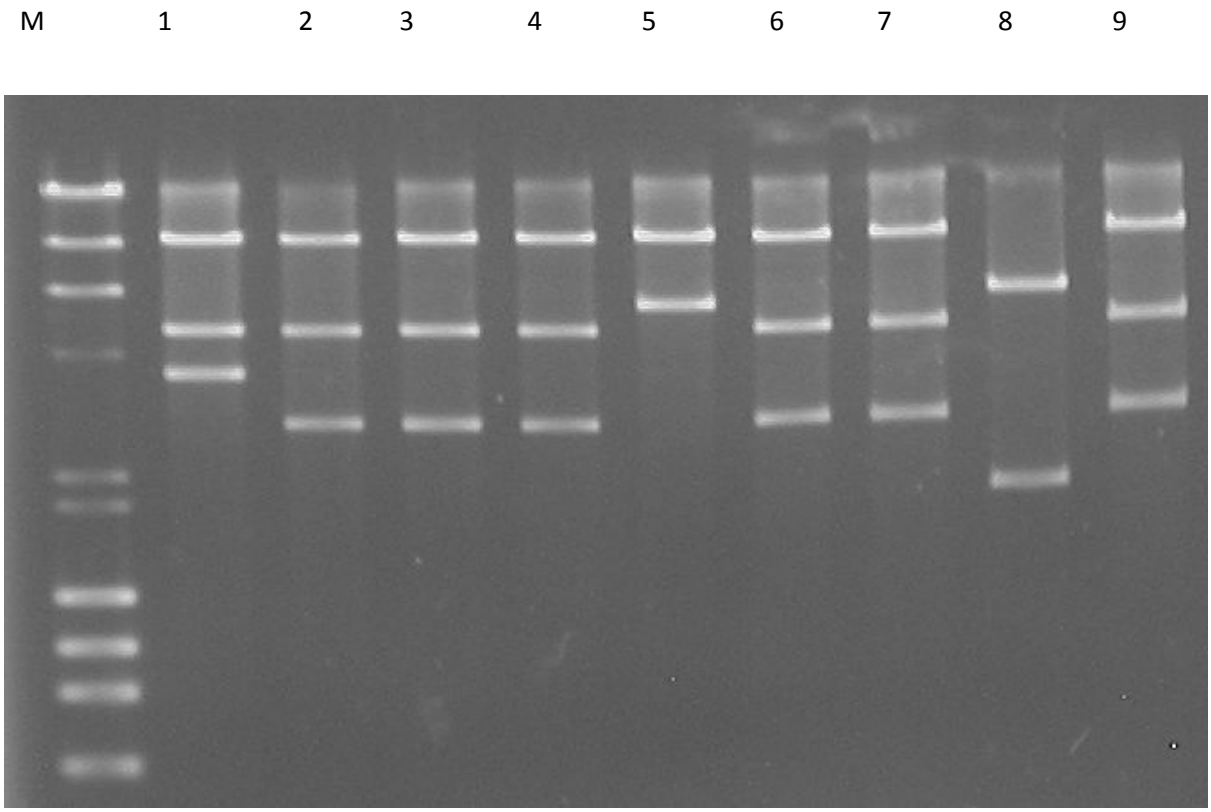


Figure 5. Electroferogram of ligation products cut by BamH1. Successful cut ligation product showed three bands (lane 2,3,4,6,7,9)

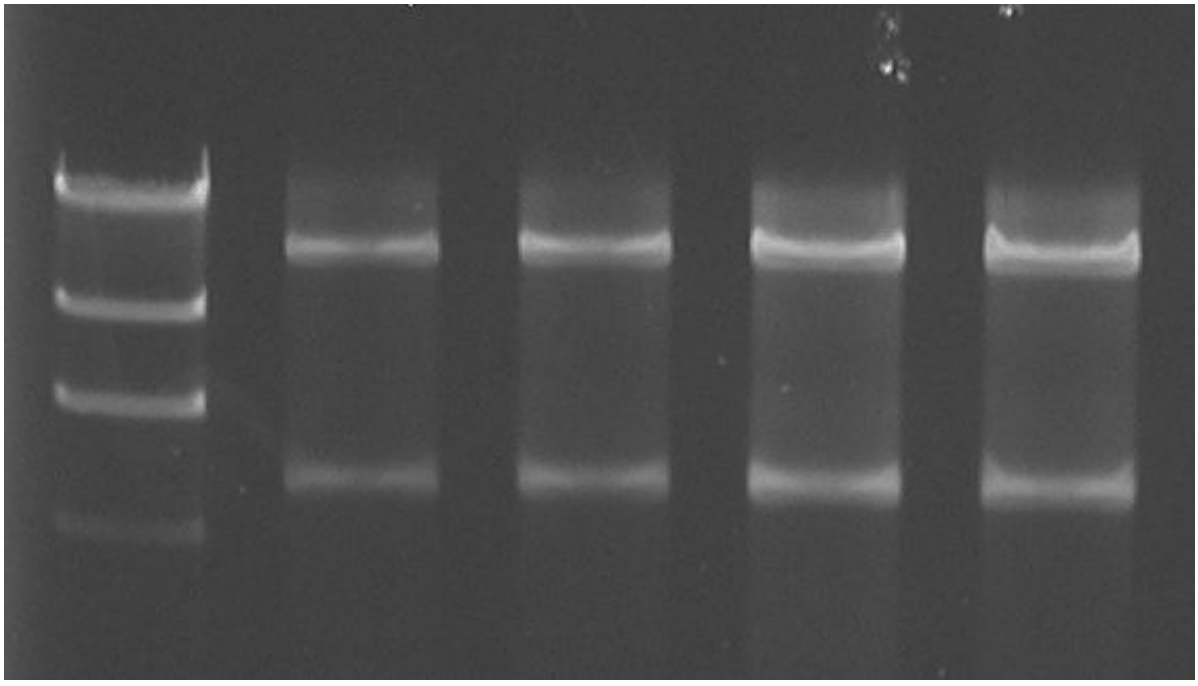


Figure 6. Electroferogram of ligation products cut by HindIII. Successful cut ligation product showed two bands.

3. Second confirmation of ligation product by PCR

In second confirmation by PCR, it was shown that wasabi defense gene, bar gene and nptII gene were existed in plasmid of ligation product (Figure 7). Transformant clones showing amplified fragments of wasabi defensin gene (0.7 kb), bar gene (0.85 kb) and nptII gene (0.7 kb).

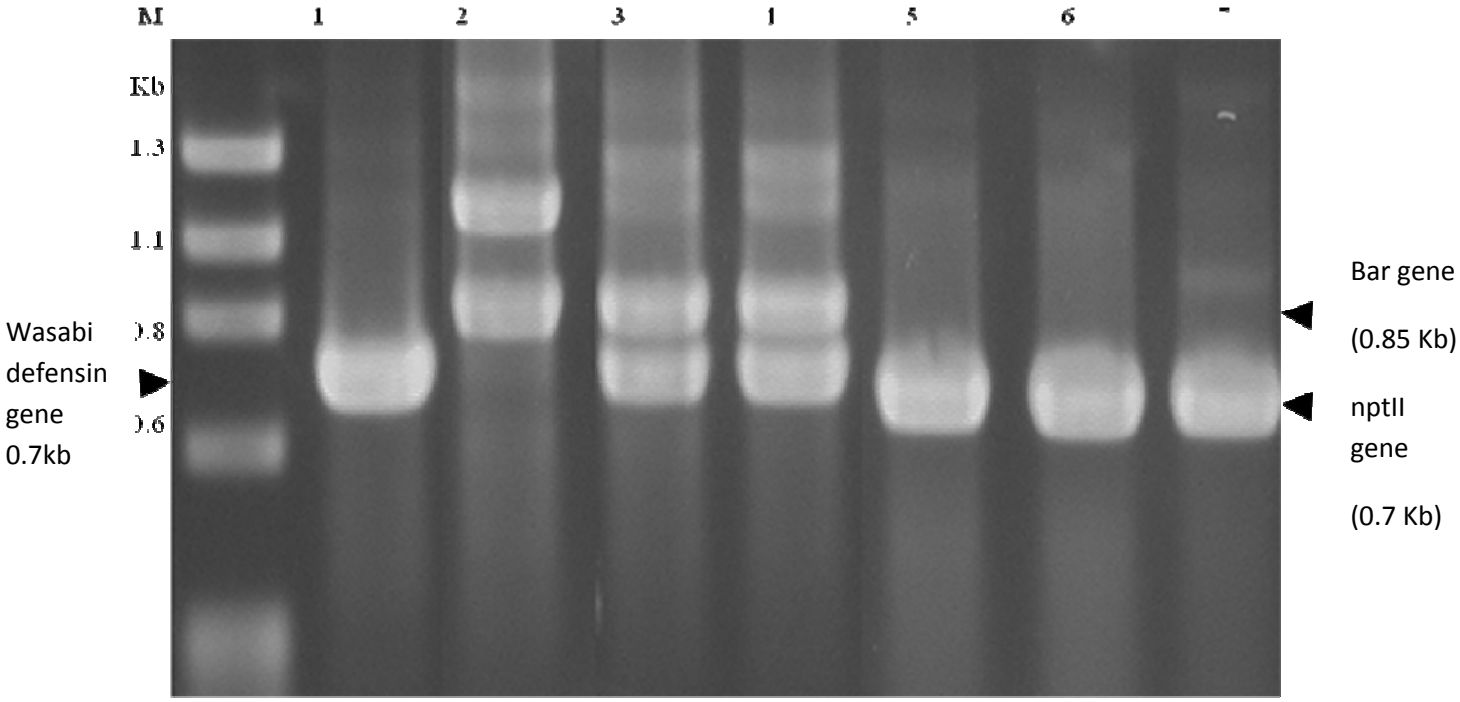
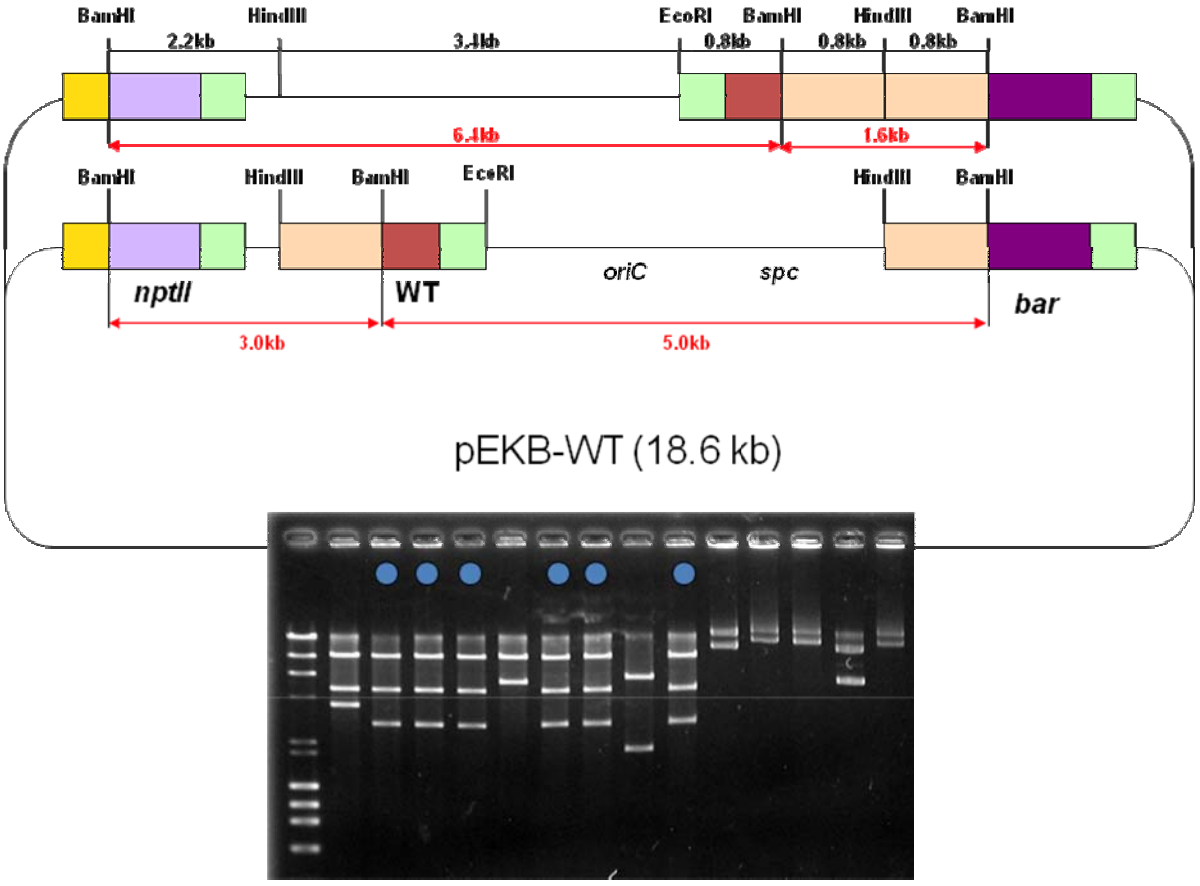


Fig. PCR analysis of wasabi defensin, *nptII* and *bar* genes in the plasmid, *pEKB/WT*. Lane M, size marker (*øX174/HaeIII* digest). Lane 1, positive control (plasmid DNA with wasabi defensin gene). Lane 2, positive control (plasmid DNA with bar gene). Lanes 3-4, Transformant clones showing amplified fragments of wasabi defensin gene (lower band) and bar gene (upper band). Lane 5, positive control (plasmid DNA with *nptII* gene). Lanes 6-⁻, transformant clones showing amplified fragment of nptII gene. Arrows indicate the amplified fragments of the introduced genes.

4. New created plasmid (pEKB-WT)

Based on the result it was concluded that the wasabi gene has been successfully cloned into the plasmid containing bar gene. Its map of plasmid of ligation product (pEKB-WT) was shown in figure 8. The result showed that the new created plasmid (pEKB-WT) was 18.6 kb and ready of use for genetic transformation of *Phalaenopsis amabilis* by biolistic and mediated by *Agrobacterium tumefaciens*.



Note : WT is Wasabi defensin gene, Bar is Bar gene

Discussion

Wasabi defensin gene is responsible for soft rot disease resistance in *Phalaenopsis* plants (Sjahril et al. (2006) and in rice (Kanzaki et al., 2002). Bar gene is responsible for herbicide resistance gene as reported by Otani et al. (2003). In this study these two important traits were cloned and will be used in genetic transformation of *Phalaenopsis amabilis* by biolistic and mediated by *Agrobacterium tumefaciens*. It is expected that the transgenic *P. amabilis* will be soft rot disease resistance as well as herbicide resistance.

Molecular cloning method performed in this study is useful for creating new plasmids for genetic transformation. Any transgenes can be inserted into binary vector EBis-KB1 (Nakamura, 2008, personal communication). Therefore, isolation of useful gene experiment would be an important purpose in the near future.

Acknowledgement

We thank to Iwate Biotechnological Institute for permission in using wasabi defensin gene in this experiment.

Reference

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