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Construction of a Binary Vector and Transformation of Tobacco with Replicase Gene from Papaya Ringspot Virus

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ABSTRACT. Papaya ringspot virus (PRV) disease is a serious disease of papaya (Carica papaya) and some of the Cucurbit plant species and, it has only been partially controlled by conventional methods. An alternative approach involves a viral coat protein (CP) transgene to generate virus resistant plants. The recent use of viral replicase gene to develop virus resistant plants has been reported for several economically important crops. This report summarizes the research efforts taken to develop virus resistant plants by the use of NIb gene from PRV.

The replicase gene of PRV was modified by introduction of start and stop codons, cloned into pSA1032 under the control of CaMV35S promoter and this expression cassette was subcloned into a T-DNA region in pSA1001 vector. The resulting NIb-binary vector was mobilized into Agrobacterium tumefaciens by conjugation. Tobacco (Nicotiana tabacum) was transformed by leaf disk transformation method yielding transgenic tobacco plants. Several randomly selected plants exhibited positive GUS expression of which successful transformation was further confirmed by PCR amplification.

INTRODUCTION

Papaya ringspot virus disease occurs in many tropical and subtropical countries worldwide leading to severe damage to papaya plantations. The pathogen is a poty virus which is the largest and economically most important plant virus group. The control of this disease is difficult because PRV is normally transmitted by several species of Aphids in a nonpersistant manner (Fitch *et al.*, 1992).

There is little genetic resistance to papaya ringspot virus in papaya germplasm. Large collection of papaya lines and cultivars representing the world's major production areas have been screened but no resistant plants have been found (Conover, 1976). Varying degrees of tolerance have been used in breeding programs. However, these conventional breeding programs are expected to compromise between useful resistance and other acceptable characters.

High level of resistance to papaya ringspot virus is known to exist in several wild *Carica* species. Interspecific hybrids between papaya and PRV resistant species have been produced with the aid of embryo rescue or ovule culture techniques (Manshardt *et al.*, 1989). Several F1 interspecific hybrids and sequidiploids produced by back crossing to

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papaya were vigorous and showed excellent field resistance to PRV. However, these plants were sterile and it seems that interspecific reproductive barrier makes the incorporation of resistant gene difficult. Cross protection, the deliberate infection of a crop with mild viral strain to limit economic damage by more virulent strains has several draw backs, including a requirement for a large scale inoculation program, reduction in crop yield and losses of cross protected plants due to super infection by virulent strains (Fitch *et al.*, 1992).

In order to overcome these problems, genetically engineered plants resistant to virus infections have been obtained by transformation of plants with wild type or modified viral genes encoding proteins. This approach was first demonstrated by Powell *et al.* (1986) by the expression of coat protein gene of virus to delay the onset of severe symptoms of tobacco. The coat protein mediated protection against virus has since been found to be effective in protecting tobacco, tomato, potato and several other plants from infection by many different viruses including papaya ringspot virus (Ling *et al.*, 1991). They have found that tobacco expressing the coat protein gene of papaya ringspot virus (Strain PRV HA 5–1 from Hawaii) showed broad spectrum resistance against several poty viruses. Since papaya is infected by only 1 poty virus, they transferred the PRV *CP* gene into tobacco and noticed that the onset of viral symptoms was delayed in plants inoculated with 3 related poty viruses such as tobacco etch virus (TEV), potato virus Y (PVY) and pepper mottle virus (PeMV). Fitch *et al.* (1992) have reported that the transgenic papaya that express *CP* gene of mild strain (HA 5–1) of the Hawaiian isolate PRV-P showed a varying degree of resistance to PRV infection.

Transgenic resistant plants with viral replicase gene have also been reported for many viruses (Golemboski *et al.*, 1990; Brederrde *et al.*, 1995; Susuki *et al.*, 1995). The mechanism behind this replicase mediated resistance has not been well understood. It will be highly beneficial if one can investigate the possibility of using replicase mediated resistance to produce resistant plants against PRV disease, since coat protein mediated protection also seems to be strain specific against PRV infection (Hautea *et al.*, 1999).

This paper describes a construction of a higher plant expression vector bearing replicase gene (*NIb*) of PRV and production of transgenic tobacco by *Agrobacterium tumefaciens* gene transfer technique. Although PRV does not infect tobacco, this well characterized transformation system was chosen to quickly assess the properties of the newly constructed vector. It might also provide insights to the replicase mediated resistance against infections by heterologous viruses such as TEV, PVY and PeMV.

MATERIALS AND METHODS

This study was conducted at the Plant Molecular Laboratory of the Institute of Science and Technology for Research and Development, Mahidol University, Thailand.

In order to obtain a functional replicase gene (*Nib*) a PCR was conducted using primers designed with start and stop codon (Forward primer- 5'CGATGGGTGGTCGTTGGCTCTTTG 3', Reverse primer- 5'CGTTACTG ATGATGAAACACATGCGT 3'). As the template DNA, the recombinant plasmid, pSA1033 which was previously constructed (Kottearachchi *et al.*, 2000) with the replicase

gene of PRV was used. The reaction mixture was composed of 2 mM $MgCl_2$, 0.2 mM dNTP, 0.6 μM primers, about 50 ng of template DNA and 5 units of *Amplitaq* polymerase (Perkin elmer). The amplification was performed in a thermal cycler (Gene amplification system 2400, Perkin elmer) programmed for 30 cycles at 95°C for 20 sec, 55°C for 25 sec, 73°C for 2 min. The amplified product was excised from 0.8% Agarose gel with TAE buffer (0.04 M Tris acetate and 0.001 M EDTA) and the DNA was purified from the gel piece by GENE CleanII[R] kit (Bio 101 Inc). The purified DNA was treated with T_4 DNA polymerase in order to eliminate the additional nucleotide at 3' end.

The pSA1032 plasmid was a plant expression vector constructed previously at the Mihidol University with the 5'untranslated region of Alfa mosaic virus (UTR) which is known as translational enhancer, between CaMV35S promoter and *NcoI* cloning site (Fig. 1). The plasmid was cut by *NcoI* restriction endonuclease and blunted with T_4 DNA

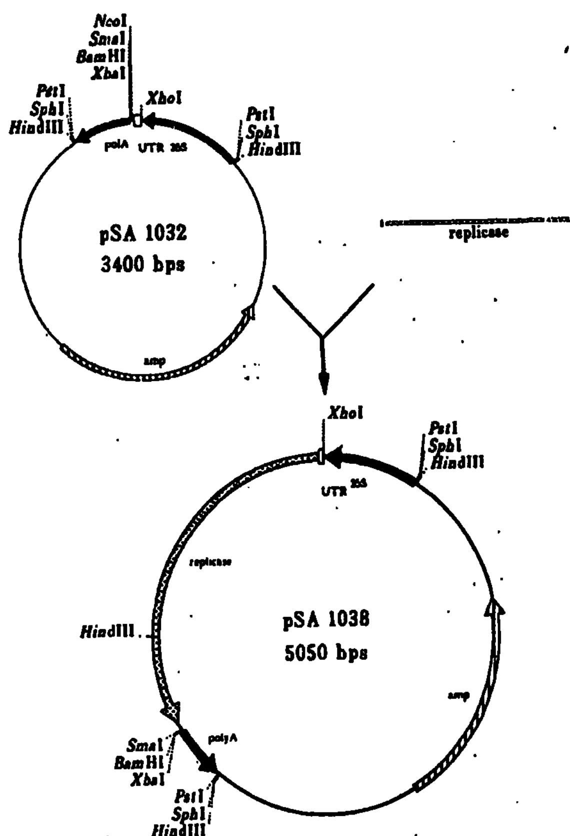

Fig. 1. Schematic representation of the construction of pSA1038 plasmid. Replicase gene (*NIb*) has been ligated at the blunted *NcoI* site of pSA1032. Correct orientation is ensured by the digestion of *HindIII* since the recognition sequence is present about 400 bp away from 3' end of the *NIb* gene.

polymerase. After purification of the modified vector and *NIb* gene, both components were ligated together and the ligated mixture was transformed into *E. coli* DH5∝ (Bethesda, 1986). The plasmid DNA was extracted from about 50 colonies and they were analysed by restriction endonuclease. The recombinant plasmids were analysed further by restriction endonucleases (Fig. 2) in order to detect the orientation of ligated product and the correctly oriented plasmid was named as pSA1038. The expression cassette was then removed by *SPhI* restriction endonuclease and the fragment was blunted by T_4 DNA polymerase. The fragment was isolated from 0.8% agarose gel with TAE buffer and purified by GENE cleanII^R kit.

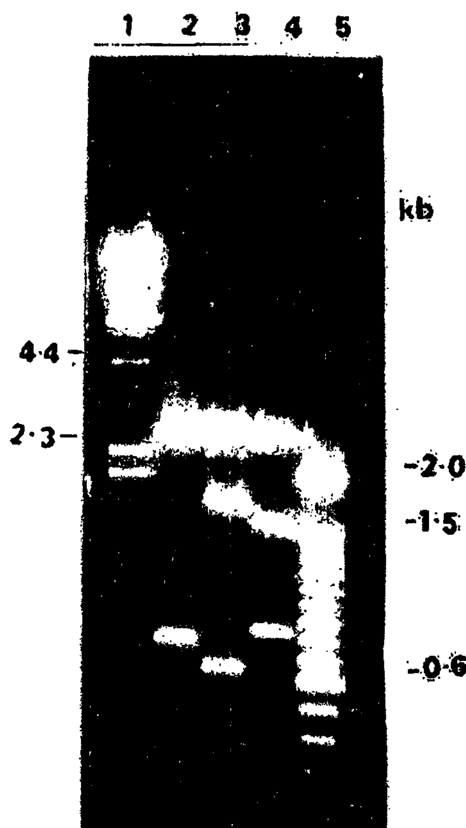

Fig. 2. Detection of the correct orientation in NIb insert. The bands were obtained by the *HindIII* digestion of pSA1032-NIb plasmids.
[Note: Lane 1- λ/*HindIII* digested DNA marker, Lane 2- pSA1032, Lane 3- Correctly oriented plasmid (pSA1038), Lane 4- Incorrectly oriented plasmid, Lane 5- 100 base pair ladder DNA marker].

The pSA1001 plasmid is a Ti-binary vector (Fig. 3). The plasmid was cut with *HindIII* restriction enzyme and blunted with T_4 polymerase. The expression cassette was ligated at the blunted *HindIII* site of pSA1001 plasmid as shown in Fig. 3. The ligated

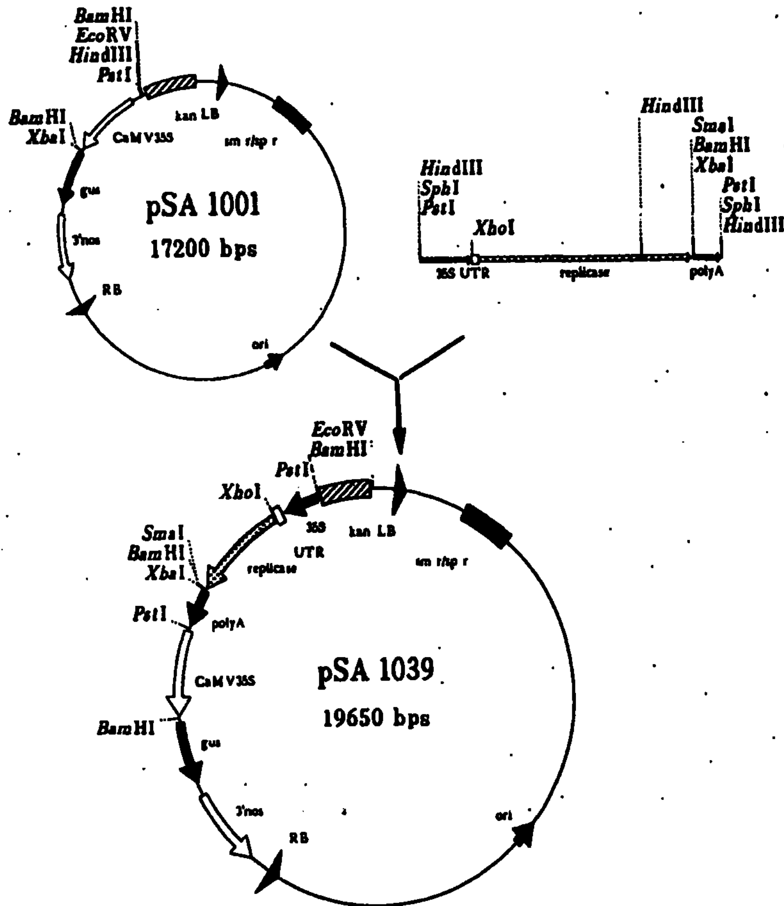

Fig. 3. Schematic representation of the construction of binary vector pSA1039. The *NIb* expression cassette was removed by *SphI* and ligated at *HindIII* site of the pSA1001 in the same direction as GUS expression cassette.

mixture was transformed into *E. coli* DH5∝ and the plasmid DNA were extracted from several colonies and analysed by restriction endonuclease to check the expected restriction pattern. Two recombinant binary vector constructed were named as pSA1039 and pSA1040. Each vector was transferred into *A. tumefaciens* strain EHA101 by means of the triparental mating system. First, the binary vector constructs were directly transferred into competent *E. coli* helper strain GJ23 which bears the mob+(pGJ28) plasmid and broad host helper (R64drd11). The transformants were selected on medium supplemented with tetracycline, kanamycin and spectinomycin that are needed to select all required plasmids. The tetracycline resistance is a marker for broad host helper (R64drd11), spectinomycin / streptomycin resistance is a marker for *NIb*-binary vector, kanamycin resistance is a marker for mob+plasmid (pGJ28). Two control plates were maintained using only GJ23

and DH5∝. In the second step, a colony of transformed *E. coli* GJ23 was mated with recipient *Agrobacterium* strain EHA101. Transconjugants were selected on medium containing rifampicin, streptomycin and spectinomycin since rifampicin resistance is an *Agrobacterium* chromosomal marker. As a control experiment, these plates were inoculated with GJ23 harbored binary construct and *Agrobacterium tumefaciens* EHA101.

The leaf pieces of *in vitro* grown *Nicotiana tabacum* cv. "petit Havana SRI" were transformed by the modified *Agrobacterium* strains carrying pSA1039 and pSA1040 plasmids as described by Mathis *et al.* (1995). Transformed calli were selected on MS (Murashige and Skoog, 1962) medium supplemented with kanamycin at a concentration of 100 mg/l and 500 mg/l cefotaxime. The selected shoots were then transferred into individual bottles containing 100 mg/l kanamycin and 250 mg/l cefotaxime. The rooted plantlets were transplanted in the autoclaved soil and grown in the green house.

The excised leaf tissues of R_0 plants were assayed for the GUS (β-Glucuronidase) expression according to Jefferson (1987). The leaf samples were incubated at 37°C overnight in 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide, 25 mM sodium phosphate buffer (pH–7.0) and 1% DMSO. In order to confirm further, PCR was conducted using genomic DNA extracted from the 2 month old putative transformed tobacco, according to the procedure described by Edwards *et al.*, 1991. The amplification was performed as mentioned earlier.

RESULTS AND DISCUSSION

PRV replicase gene is encoded as a part of poly protein and the message does not contain the 5' untranslated region or even the translational initiation codon. The transcriptional element was added by cloning the PRV *NIb* gene into the pSA1032 vector which was previously made with the alfa mosaic virus untranslated region (UTR). The translational initiation and termination codons were added by modified PCR primers. In the resulting product, *NIb* gene contains ATG as the initiation codon and UAA as the termination codon. Subsequently this gene was inserted between the alfa mosaic virus UTR which is present next to CaMV35S promoter and poly A terminator. The Fig. 2 illustrates the 2 possible orientations that the insert has been ligated. The correctly oriented plasmid was identified as the plasmid shown in lane 3 since it gave the exact restriction pattern expected by *HindIII* enzyme.

The pSA1001 plasmid has a cloning site at *HindIII*, inside the T-DNA region. The GUS and kanamycin resistant expression cassettes also are present inside the border sequence of the T-DNA unit. As described earlier, replicase expression cassette was removed by *SphI* and ligated at *HindIII* location of pSA1001. The final plasmid construction shown in Fig. 3. When the recombinant plasmid is cut by *BamHI*, two possible ligation events could be identified. In one, plasmid *NIb* expression cassette has been ligated in the same direction as GUS expression cassette (pSA1039) while the other was opposite to the GUS (pSA1040) (Fig. 4).

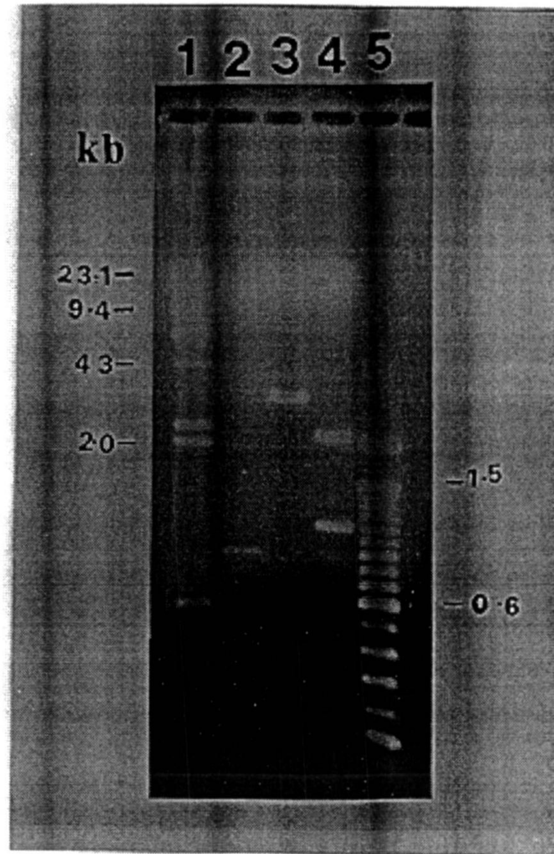

Fig. 4. Analysis of pSA1001-*NIb* plasmids by *BamHI*.
[Note: Lane 1- λ/*HindIII* digested DNA marker, Lane 2- pSA1001 plasmid, Lane 3- pSA1040 plasmid, Lane 4- pSA1039 plasmid, Lane 5- 100 base pair ladder DNA marker].

The recombinant binary plasmids were then used to transform the avirulent *Agrobacterium* strain EHA101 using the tri-parental procedure. Since rifampicin resistance is an *Agrobacterium* chromosomal marker and spectinomycin and streptomycin resistance are markers for binary vector, only the conjugated *Agrobacterium* which possess binary vectors could be grown on this medium. The control plates were not able to be cultured since they lacked particular plasmids. Therefore, an isolate of EHA101 carrying pSA1039 and another isolate carrying pSA1040 were conserved for plant transformation.

The transformation of tobacco was conducted by leaf disk transformation method which is simple and straight-forward. If tobacco could be transformed and could express the selectable and reporter genes, it proves that the *Agrobacterium-NIb*-binary vector system works positively and therefore, this system can be used to transform other plants which have complicated regeneration systems. Shoots started to appear about 3 weeks after

inoculation. Negative control explants that were maintained without Ti binary vector were not able to produce any shoots under the level of 100 mg/l kanamycin. To check the regeneration ability of the medium, a positive control plate was maintained without kanamycin (Fig. 5). Only 1 shoot from each callus piece was taken in order to ensure that all shoots represent a separate transformation event. About 50 kanamycin resistant plants were able to produce roots under 100 mg/l kanamycin. The T-DNA region contains a plant expressible bacterial derived neomycin phosphotransferase II (*NPT II*) gene which upon transfer, genome integration and expression in plant tissues confers resistance to the antibiotic kanamycin. Therefore, the shoots derived from the medium containing 100 mg/l kanamycin indicates that at least that part of the T-DNA unit has been integrated into tobacco genome.

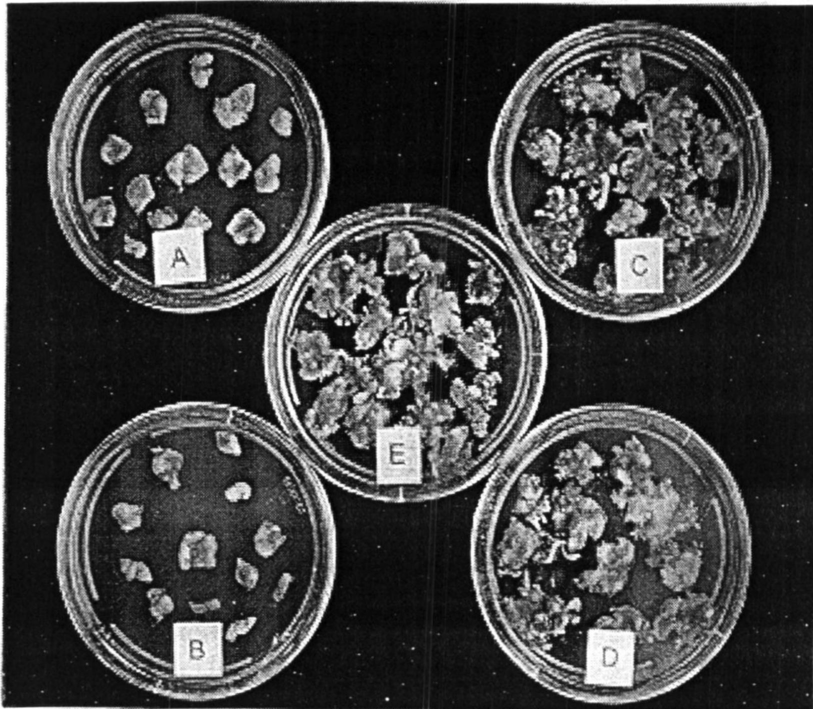

Fig. 5. **Explants of tobacco cultured on selectable medium with 100 mg/l kanamycin.** [Note: A- uninoculated control explants, B- control explants inoculated with *A. tumefaciens* EHA101 without binary vector, C- control explants inoculated with *A. tumefaciens* EHA101 with pSA1039, D- explants inoculated with *A. tumefaciens* EHA101 with pSA1040, E- control explants cultured on medium without kanamycin (cultures were photographed 5 weeks after inoculation)].

The β-Glucuronidase catalyses the hydrolysis of substrate X-Gluc and liberates a molecule of 5-bromo-4-chloro-indoxyl (XH) which produces blue colouration in the presence of oxygen. The T-DNA unit of the plasmid pSA1039 and pSA1040 contain *GUS* gene and upon integration into plant genome, it expressed the β-Glucuronidase enzyme which was easily detectable as described by Jefferson (1987). In the rooting medium

where the explants were grown large enough to remove a leaf from each, 6 plants were analysed for the presence of T-DNA by *GUS* histochemical staining. Five *GUS* positive plants were detected although the intensity of blue colouration was different among them. This might most probably be due to the positional effect of T-DNA integration and different number of insertions. The β-glucuronidase is generally absent in most plant species and it is a reliable technique for the confirmation of gene transformation (Hull *et al.*, 1995).

Since the *NIb* gene is flanked by *GUS* and *NPTII* genes the *GUS* activity and kanamycin resistance ensures the presence of *NIb* gene. Further confirmation was done by PCR amplification which was accomplished using oligonucleotide primers that were previously designed for the isolation of *Nib* gene of PRV as shown in Fig. 6. The

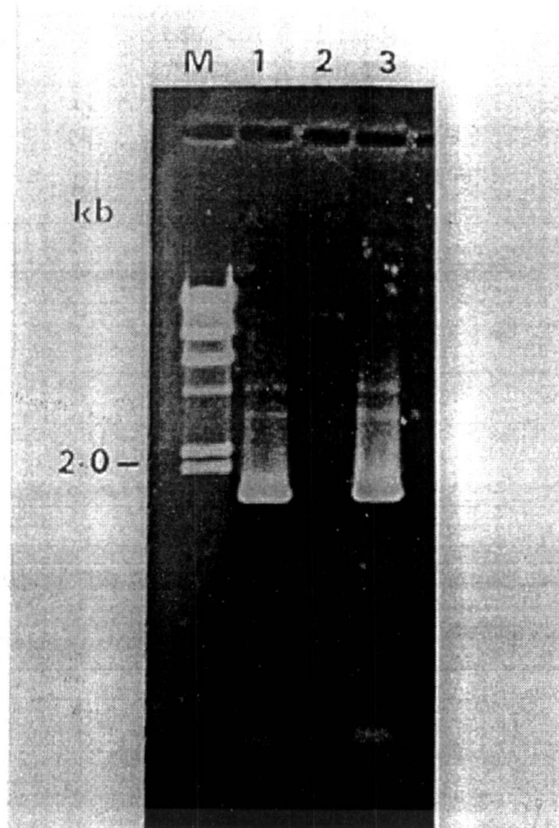

Fig. 6. PCR analysis of genomic DNA from transgenic tobacco plants.
[Note: Lane M- λ/*HindIII* digested DNA marker, 1- PCR product obtained from genomic DNA of plant line T5 (transformed with pSA1039), 2- Negative control conducted using untransformed genomic DNA of tobacco, 3- PCR product obtained from genomic DNA of plant line T12 (transformed with pSA1040)].

expressible form of *NIb* gene was flanked by *GUS* and *NPTII* genes in the T-DNA unit of pSA1039 and pSA1040. The amplification was found corresponding to the exact size of *NIb* gene which was 1.6 kb. All these data confirm that *Nicotiana tabacum* has been transformed by the replicase gene of PRV by *NIb*-binary-*Agrobacterium* system. Therefore, this well characterized system can later be used to transform *Carica papaya* and other PRV infected species. Ling *et al.* (1991) has reported the protection against other poty viruses such as TEV, PeMV and PYV from PRV coat protein gene in transgenic tobacco. Therefore, further experiments will be conducted to test whether the transgenic tobacco produced from the *NIb* gene of PRV is resistant to the infections of those poty viruses.

CONCLUSIONS

The replicase gene of papaya ringspot virus (Thai isolate) was modified by adding start and stop codons by polymerase chain reaction. The gene was then cloned into pSA1032 plasmid under the control of CaMV35S promoter. Two Ti binary vectors (pSA1039 and pSA1040) were constructed bearing *NIb* expression cassette for *Agrobacterium tumefaciens* mediated gene transformation. The *NIb*-binary vectors were mobilized into *Agrobacterium tumefaciens* by conjugation. *Nicotiana tabacum* was successfully transformed by *A. tumefacies-NIb*-binary vector system with leaf disk transformation method. Transformants were selected on MS medium containing 100 mg/l kanamycin concentration. Several *GUS* positive plants were detected and transformants were confirmed further by polymerase chain reaction.

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