



## **CHAPTER FOUR**

### **TRANSFORMATION OF *ARABIDOPSIS npr1-2* MUTANT WITH BANANA *NPRI*-LIKE CODING SEQUENCES**

#### 4.1 Abstract

The full length coding regions of the banana *NPR1*-like genes (*MNPR1A*, or *MNPR1B*) were cloned into plasmid *pBin19-LBR* where their expression was regulated by the control of the Cauliflower mosaic virus 35S (35S CaMV) promoter and terminator sequences. These plasmids were introduced into *Agrobacterium tumefaciens* LBA4404 cells and used to transform *npr1-2 Arabidopsis* mutant plants. Stable gene integration of the two coding sequences in the mutant plants was confirmed by PCR analysis and sequencing and, expression of the introduced genes was validated by qRT-PCR. During the first selection stage, of more than 1000 T-1 seeds from putative transformed plants screened on Murashig and skoog (MS) medium supplemented with kanamycin, a transgenic plant recovery rate of about 16-20% was obtained. However, following PCR analysis with genomic DNA from leaves of these plants, only between 16-18% of these regenerants had the gene integrated within their genome. These were considered as “true” transformants. During the second selection step (T-2 selection) of seeds, about 50% of seeds regenerated on kanamycin. While during the T-3 selection, more than 65% of seeds survived on the kanamycin-containing medium and at the T-4 selection stage more than 95% of positive transformants were obtained which were considered as homozygous and used for further analysis. Gene expression analysis of the two banana *NPR1* genes and the *Arabidopsis PR-1* gene in selected homozygous transgenic lines showed that transcription of the banana *NPR1* genes did not alter transcript amounts of the *Arabidopsis PR-1* gene.

## 4.2 Introduction

Rapid advances in plant molecular biology have been facilitated by the discovery of organisms, such as *Agrobacterium tumefaciens*, that can be engineered and used for gene transfer into plant genomes, facilitating expression of transgenes and evaluation of their function (Bevan, 1984; Gelvin, 2003). Binary vectors are essential tools in *Agrobacterium*-mediated transformation systems (Bevan, 1984) comprising a transfer- (T)-DNA region and the vector's backbone. The T-DNA region in most engineered binary vectors like *pBin19* is delimited by left and right border (LB, RB) sequences and contains a complete plant selection marker expression cassette and a multiple cloning site (MCS) to facilitate introduction of the desired expression cassettes; reporter genes such as the *lacZ* gene which encode beta- ( $\beta$ )-galactosidase enzyme and whose expression can easily be monitored for screening of transformed cells. The vector backbone on the other hand has selectable marker genes for the bacteria and replication functions enabling it to replicate in both *Agrobacterium* and *Escherichia coli* (*E. coli*) cells (Bevan, 1984; Komori *et al.*, 2007). As early as 1987, researchers started using the *Agrobacterium* system for the transformation of *Arabidopsis*. The initial procedure involved applying *Agrobacterium* inoculum directly onto seeds, growing plants using these seeds, harvesting of progeny before selection on an antibiotic medium (Feldman and Marks, 1987). Chang *et al.* (1994) have employed the "clip 'n squirt" method which entails clipping off the reproductive inflorescences from *Arabidopsis* plants before application of the inoculum onto the rosettes. For higher transformation efficiency, the procedure was repeated after the emergence of secondary inflorescences on the plant. Seeds that developed from these inoculated plants were selected on medium containing the appropriate antibiotic. Other *Arabidopsis* transformation approaches include a tissue culture (Valvekens *et*

*al.*, 1988), vacuum infiltration (Bechtold *et al.*, 1993) and the most commonly used floral dip (Clough and Bent, 1998) methods.

The objective of this part of the study was to stably intergrate two previously isolated banana *NPRI*-like coding sequences (*MNPRIA* and *MNPRIB*; Endah *et al.*, 2008) into *Arabidopsis npr1-2* mutant plants. There are four commonly used *Arabidopsis npr1* allelic mutants with different positional mutations within the *NPRI* gene sequence (Cao *et al.*, 1997). Unlike the *npr1-1 Arabidopsis* mutant which has a mutated histidine at position 334, the *npr1-2* results from a mutation at position 150 from a cysteine to a tyrosine. *Arabidopsis npr1-2* mutant plants were used due to availability at the time of the study.

### 4.3 Materials and methods

#### 4.3.1 Gene cassette design

##### 4.3.1.1 *Plasmids*

p*MNPR1A* and p*MNPR1B* (-70°C glycerol stocks; FABI, South Africa) harbouring the banana *MNPR1A* and *MNPR1B* coding region respectively in the PCR®4-TOPO® vector backbone were streaked in 90-mm Petri dishes containing 20 mL of Luria broth (LB) agar medium [1% bacto-tryptone (w/v), 0.5% bacto-yeast extract (w/v), 1% sodium chloride (w/v) and 1.2% bacterio- agar (w/v) pH 7.5]. The medium was supplemented with 50 mgL<sup>-1</sup> carbenicillin for plasmid selection. Petri dishes containing plasmids were incubated at 37°C overnight. Resulting single white colonies from overnight plates were individually cultured in 15 mL Falcon tubes containing sterilized 5 mL of liquid LB medium [1% bacto-tryptone (w/v), 0.5% bacto-yeast extract (w/v) and 1% sodium chloride (w/v); pH 7.5]. Liquid cultures were supplemented with the same antibiotic as above before overnight incubation at 37°C with continuous shaking at 200 revolutions per minute (rpm).

The binary vector p*Bin19-LBR* (Fig. 4.1) containing a double 35S CaMV promoter and 35S CaMV terminator sequences was used as a host for the banana *NPRI* genes to facilitate transformation into *A. tumefaciens*. Plasmid p*Bin19-LBR* was also obtained from a -70°C glycerol stock (FABI, South Africa) and cultured as described above for the p*MNPR1* plasmids with 50 mgL<sup>-1</sup> of kanamycin added to the medium.

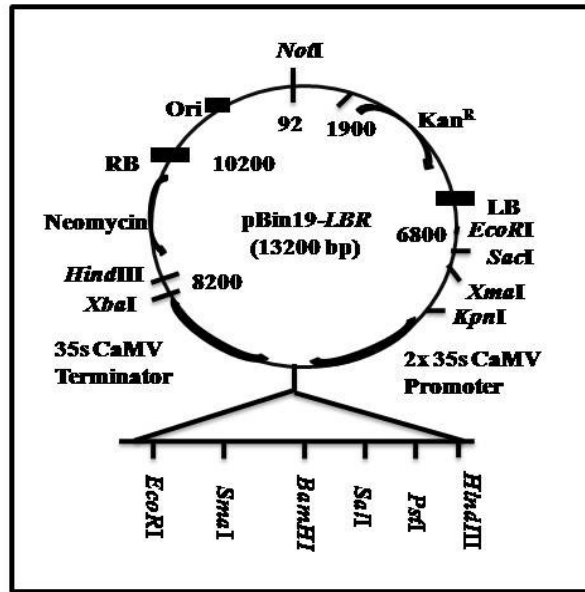


Figure 4.1 pBin19-LBR vector map.

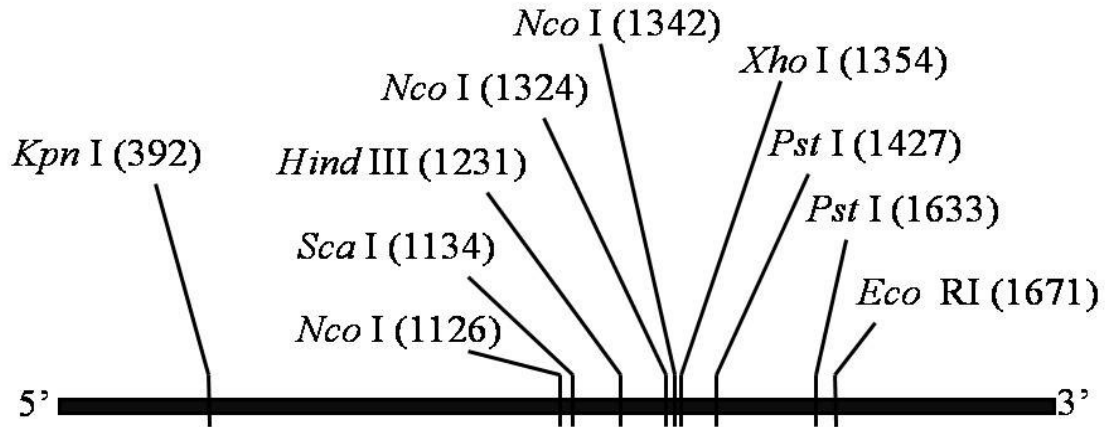
#### 4.3.1.2 *MNPR1* cloning

Plasmid DNA was isolated from p*MNPR1* cells using the GeneJet™ plasmid miniprep kit (Fermentas, Canada) according to the manufacturer's instructions. Primers (Table 4.1) with additional *Bam* HI, *Sma* I and *Xba* I restriction sites were designed to flank the 5' and 3' ends of the *MNPR1* coding sequences (Fig. 4.2). Designed primers were used to amplify the entire *MNPR1* coding regions which were then cloned into the plasmid p*Bin19-LBR*.

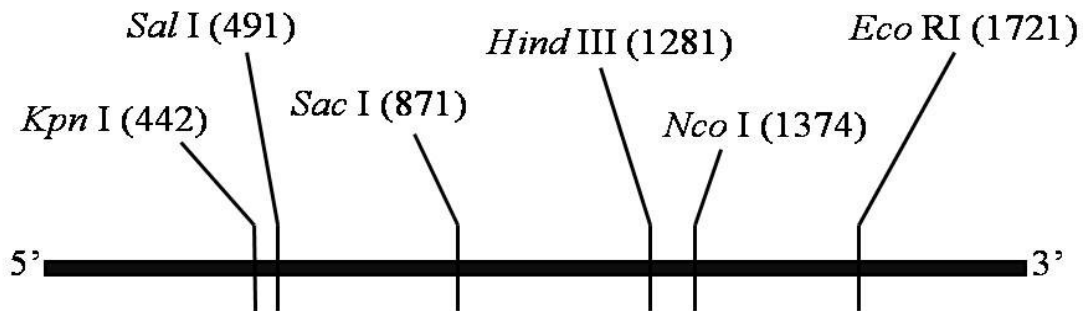
**Table 4.1** Primer sequences for the amplification of the *MNPR1* coding region.

Primer name	Primer sequence
MNPR1A_FF	TAGGGATCCATATGGAAGACAAC
MNPR1A_RR	TAGTCTAGACCCGGGATACAGCACAAT
MNPR1B_FF	TAGGGATCCACACGGCGGGACATG
MNPR1B_RR	TAGTCTAGACCCGGGGGTATAGAATCG

*MNPR1A* (1927 bp)



*MNPR1B* (2073 bp)



**Figure 4.2** Selected restriction sites within the *MNPR1A* and *MNPR1B* coding region.

The PCR reaction was set up using the Expand long-template PCR reaction kit (Roche Diagnostics, UK) with 50 ng of plasmid DNA and following instructions recommended by the supplier. The reaction was carried out by initially denaturing the DNA at 94°C for 2 min followed by a cycling step consisting of 10 cycles with DNA denaturation at 94°C for 10 s, primer annealing at 64°C for 30 s and DNA elongation at 68°C for 1 min. This was followed by a second cycling step of 15 cycles under the same conditions as the first although during this stage, the elongation phase was increased by 10s/cycle. Final DNA elongation occurred at 68°C for 10 min.

PCR products were separated by electrophoresis on a 1% (w/v) denaturing low LE agarose gel (Promega, South Africa) and a 1.8 kb *MNPR1* fragment excised from the gel. The DNA from the gel was recovered using the DNA clean and concentrator<sup>TM</sup> kit (Zymo Research, USA) as recommended by the supplier. Both *MNPR1* DNA fragments (400 ng) were then individually ligated in a 10 µL reaction containing 55 ng of the p*Gem-T-easy* plasmid (Promega, UK) to which a 2X ligation buffer and 10 U of T<sub>4</sub> DNA ligase (Roche Diagnostic, UK) were added. Reactions were incubated overnight at 4°C and used for transformation of DH5α<sup>TM</sup>-T1<sup>R</sup> competent cells (Invitrogen, UK).

For transformation of *E. coli* competent cells, ligation mixture (6 µL) and competent cells (25 µL) were incubated for 30 min on ice followed by heat shock treatment of cells at 42°C for 45 s. The samples were immediately placed on ice for 2 min prior to the addition of 0.2 mL of SOC medium (20% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM 10 mM MgSO<sub>4</sub>, 20 mM glucose, 10 mM MgCl<sub>2</sub>). For rapid replication of cells, samples were incubated with

continuous shaking at 200 rpm for 1 h at 37°C after which 0.1 mL of the transformation reaction was spread on LB agar-containing Petri dishes supplemented with 50 mgL<sup>-1</sup> carbenicillin. The Petri dishes were then incubated at 37°C overnight and 10 white *E. coli* colonies from each plate were individually cultured in 5 mL of liquid broth supplemented with 50 mgL<sup>-1</sup> carbenicillin. Samples were cultured overnight on a shaker at 200 rpm and 37°C. To confirm presence of the ligated *MNPR1* gene fragments and orientation of the fragments within the *pGem-T\_easy* plasmid, plasmid DNAs isolated from overnight cultures were sent for sequencing and, the plasmids with the correct *MNPR1* sequences used for cloning of the genes into the binary vector. Plasmids selected for subsequent downstream reactions were named *pGem-MNPR1A-3* and *pGem-MNPR1B-4*.

For cloning into *pBin19-LBR*, 300 ng of plasmid DNA from *pGem-MNPR1A-3* and *pGem-MNPR1B-3* was digested with 10 U of each *Bam* HI and *Sma* I (Fermentas, Canada) in a 20 µL reaction mixture containing the required buffer as recommended by the supplier. Digestion of plasmid DNA was carried out at 37°C for 1 hr in a 1.5 mL Eppendorf tube. The resulting DNA fragments were separated by electrophoresis on a 1% agarose gel. The 1.8 kb fragment of interest was excised from the gel and the DNA recovered as described above.

A parallel digestion was also carried out using DNA from plasmid *pBin19-LBR* under the same conditions as described above. Five µL of the digest was separated by electrophoresis on a 1% agarose gel to verify that the plasmid had been completely linearized. The remaining *pBin19-LBR* digest was purified using the phenol: chloroform purification method (Sambrook *et al.*, 1989; Sambrook and Russell, 2001). The entire linearized and purified *pBin19-LBR* plasmid

DNA was dephosphorylated using alkaline phosphatase enzyme and alkaline phosphatase buffer (Fermentas, Canada) as instructed by the manufacturer. Dephosphorylation of the plasmid DNA was carried out at 37°C for 1 hr. After dephosphorylation, the DNA was again purified using the phenol: chloroform purification method as described above. The pellet obtained at this stage was re-suspended in 20 µL of sdH<sub>2</sub>O.

The gel purified *MNPR1* fragments were ligated into dephosphorylated *pBin19-LBR* and this ligation mixture was used to transform competent DH5αTM-T1<sup>R</sup> cells as described above. Antibiotic selection of transformed cells was done on LB using 50 mgL<sup>-1</sup> kanamycin in the medium. Isolated plasmid DNA from cells of 10 white *E. coli* colonies was analysed by restriction enzyme digestion and plasmid DNA sequencing. A glycerol stock was prepared from positively identified samples and stored at -70°C. Plasmids were designated: *pLBR-MNPR1A* and *pLBR-MNPR1B* (Fig. 4.3).

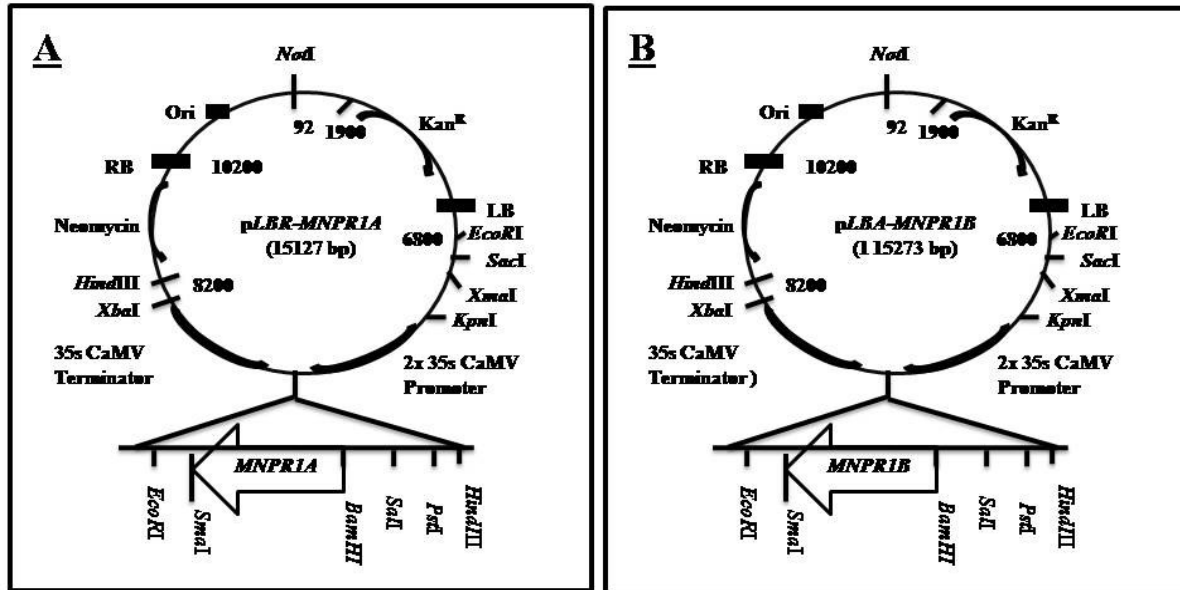


Figure 4.3 Plasmid maps of *pLBR:MNPR1* and *pLBR:MNPR1B*.

#### 4.3.2 Agrobacterium transformation

For *Agrobacterium* transformation, three gene cassettes were used. These consisted of plasmid: pLBR-MNPR1A and pLBR-MNPR1B, with plasmid pBin19-LBR serving as the control. Each of these plasmids was individually transformed into *Agrobacterium* LBA4404 competent cells using the freeze thaw method as described by Chen *et al.* (1994). Positive transformants were selected after plasmid restriction enzyme digestion. Resultant *Agrobacterium* strains harbouring the plasmids were designated: pLBA-MNPR1A, pLBA-MNPR1B and pLBA-LBR.

#### 4.3.3 Transformation of *npr1-2 Arabidopsis* mutants

##### 4.3.3.1 *Growth of npr1-2 Arabidopsis mutant plants*

*Arabidopsis npr1-2* mutant seeds (donated by Dr. Xinnian Dong, USA) were rinsed in 70% ethanol for 1 min followed by surface sterilization in 1.5% sodium hypochlorite (v/v) for 20 min at room temperature. Seeds were thoroughly rinsed (four times) using distilled water (dH<sub>2</sub>O) and re-suspended in 0.1% agar (w/v). Seeds were plated onto 20 mL Murashige and Skoog (MS) containing agar plates (4.5 gL<sup>-1</sup> MS salt, 20 gL<sup>-1</sup> sucrose and 12 gL<sup>-1</sup> Bacterio-agar). Seeds in plates were stratified for 4 days at 4°C in the dark and thereafter, exposed to a 16 h light / 8 h dark regime cycle for one week. Healthy growing seedlings were potted in moist 44-mm Jiffy-7 pots (Jiffy International AS, Norway). The Jiffy pots (45 each) were placed in 40 x 30 x 9 cm trays without holes to facilitate sub-irrigation. Trays were covered with cling wraps to ensure for maximum humidity. These were then transferred to controlled environment growth chambers.

Each chamber was provided a photosynthetic photon flux of  $200 \mu\text{mol. m}^{-2} \text{s}^{-1}$ , a photoperiod of 16 h day/8 h dark regime and a relative humidity of  $75 \pm 5\%$ . The cling wraps were removed from the trays after 2 weeks when the plants had developed at least 2 true leaves. Plants were then maintained under the above mentioned growth conditions until the formation of inflorescences. Primary inflorescences were cut off from the plants to facilitate the formation of secondary inflorescences. Plants in which most of the inflorescences were still closed were used for transformation via floral dipping into an *Agrobacterium* inoculum.

#### 4.3.3.2 *Plant transformation*

*Agrobacterium* solutions used for plant transformation were prepared from *pLBA-MNPR1A*, *pLBA-MNPR1B* and *pLBA-LBR* cultures which had been grown in liquid LB broth up to an optical density ( $\text{OD}_{600 \text{ nm}}$ ) of between 0.8-1.0. These inocula (100 mL) were centrifuged at room temperature for 10 min at 10 000 rpm. The pellets were re-suspended in 100 mL of a 5% sucrose solution (w/v) supplemented with 0.05% Silwett L-77 (v/v; Lehle seeds, USA). Plants were transformed following a modified method of the floral dip technique as described in Clough and Bent (1998). For this, the inflorescences of each plant were dipped in the respective inoculums for 10 s. After dipping plants were placed in a horizontal position in the trays and the trays were covered with foil paper to ensure for optimum humidity. The following day, foil papers were removed from the trays and the plants were placed in an upright position. Floral dipping was repeated twice over a 2 week period to optimize the transformation efficiency and using freshly prepared inoculums during each process. The plants transformed were designated as *npr1:MNPR1A*, *npr1:MNPR1B* and *npr1:35S CAMV*.

#### 4.3.3.3 *Screening of transformed lines*

Selection of *npr1* transgenic lines harbouring either the *pLBA-MNPR1A*, *pLBA-MNPR1B* and *pLBA-LBR* gene cassette were performed under sterile conditions on MS plates supplemented with 50 mgL<sup>-1</sup> of kanamycin. Seedlings that germinated and formed roots in the kanamycin containing medium were potted into jiffy pots and grown until seed formation as described previously. The selection process was carried out over four generations (involving successive kanamycin selection, potting, seed formation and harvesting steps) until homozygous lines were obtained. Homozygous lines consisted of plants where by more than 80% of their seeds could vigorously regenerate on kanamycin-containing plates. After the first selection step, plantlets were potted, appropriately labelled and treated as independent transformation events. Seeds that were subsequently harvested from these independent lines were also appropriately labelled and treated separately. During each selection process, leaf material was harvested from the potted plants for DNA isolation and/or cDNA synthesis for verification of gene integration via PCR. Seeds were always harvested from a total of 10 individual plants in which the gene of interest had been amplified.

#### 4.3.4 Transcription measurement

*MNPR1A*, *MNPR1B* and *PR-1* transcript level of five to six independent transformation events were determined by quantitative real time PCR (qRT-PCR) using RNA extracted from 5-wk-old leaves from homozygous transgenic lines. Leaf material for RNA extraction was also collected from non-transgenic wild-type (Wt) and *npr1* mutant *Arabidopsis* plants grown in parallel to the

homozygous transgenic lines to serve as controls. Total RNA extraction in all samples to be analyzed was performed using the Trizol reagent (Invitrogen Life Technologies, San Diego, California, USA) and following the manufacturer's instructions. Contaminants in the extracted RNA, particularly DNA, were eliminated by treating RNA samples with TurboDNA free reagent (Ambion, UK) according to steps recommended by the manufacturer. First-strand complementary DNA (cDNA) was synthesized using 2 µg of the treated RNA using the superscript II first strand cDNA synthesis kit (Invitrogen Life Technologies, San Diego, California, USA) and random hexamer primers (Invitrogen, UK) as instructed by the manufacturer.

The Netprimer3 program (Premier Biosoft, Palo Alto, CA, USA) was used to design primers for the three target genes *MNPR1A* (DQ925843), *MNPR1B* (EF137717) and also *AtPR-1* (NM\_127025-2). The endogenous control primer was designed from the gene encoding the *Arabidopsis* actin protein (At3g18780). All primers were designed to have a GC content of between 50-55% consisting of 20-22 nucleotides and to amplify a product of between 80-150 bp.

Prior to the selection of primers to be used, the quality of cDNA and the efficiency of each primer pair were verified by conducting a conventional PCR reaction and detecting amplified DNA products on a 2% agarose gel (w/v) to confirm the correct size of the DNA product. Primers (Table 4.2) which produced the desired single band product were further assessed for their efficiency by conducting a qRT-PCR experiment with a serial dilution (0.5, 0.25, 0.125 and 0.1) of cDNA.

**Table 4.2** Primer sequences for determining transcripts of the *Arabidopsis PR-1* and banana *NPR1* genes in *Arabidopsis*.

Primer name	Primer sequence
qMNPR1A_FF	GCTCGACAGATTTTGCTTATC
qMNPR1A_RR	GGAAGACAAAGCAGACTTGTTG
qMNPR1B_FF	CCTCCTTGGTATTCTGGACA
qMNPR1B_RR	CTTCTTCTCTAGGGTGATGG
qPR1_FF	CGGAGCTACGCAGAACAAC
qPR1_RR	CTCGCTAACCACATGTTCA
qActin_FF	AGTGGTCGTACAACCGGTATTGT
qActin_RR	GATGGCATGGAGGAAGAGAGAAAC

The Sybrgreen master mix (Roche Diagnostics, UK) was used for the qRT-PCR as follows: 5  $\mu$ L of cDNA template, 1  $\mu$ L of 10 $\mu$ M primers, 10  $\mu$ L SYBR-Green I master mix and 3  $\mu$ L nuclease-free water were added into respective wells in a 96 well-RT-PCR micro titer plate. Non-template control reactions containing water instead of cDNA as template were included in the reaction. The qRT-PCR reaction was set up as follows: denaturation for 10 min at 95°C, followed by an amplification phase of 40 cycles consisting of a DNA denaturation step at 94°C for 5 s, primer annealing at 60°C for 5 s and DNA extension at 72°C for 10 s. The reaction was finally subjected to a temperature of 65°C for 10 s and the fluorescent signal for each sample was assessed at 95°C.

A standard curve for each gene was also established using serial dilutions of the cDNA to a final concentration of 1, 0.5, 0.25, 0.125, 0.0625, 0.03125 and 0.015625 of the original concentration. For each primer set, a non-template control (NTC) reaction containing water as a template was included and, each reaction was carried out in triplicates. The experiment was repeated twice with a different set of independent plants and leaf material was sampled identically as described above. By use of the manufacturer's software program, the relative transcript amount in each sample was determined. The Applied Biosystems, User Bulletin No. 2, (2000) and steps described in Livak and Schmittgen (2001) were used for data analysis. The basal relative transcript amount for each sample was expressed relative to the actin gene (Applied Biosystems, User bulletin No.2, 2001; and steps outlined chapter 2 of this thesis).

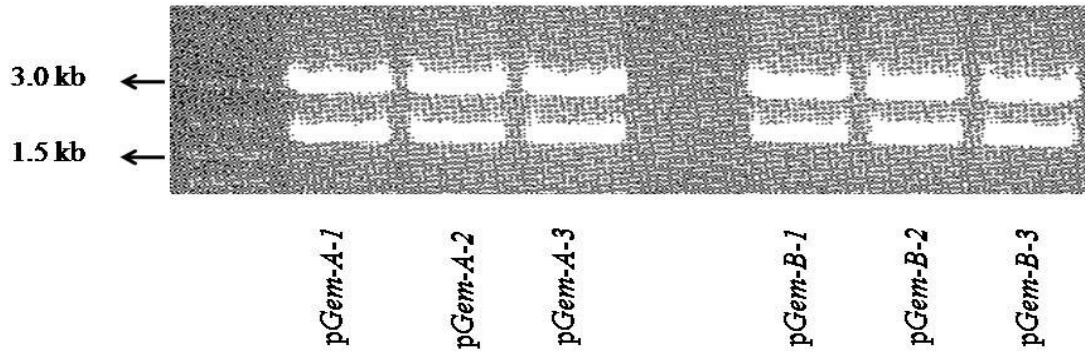
#### 4.3.5 Statistical analysis

To analyze differences in transcripts amounts the statistical analysis software-SAS<sup>®</sup> (SAS, USA) was used together with a Student's T-Test. The cut-of value was set at  $P < 0.05$  and significant differences among samples was determined using ANNOVA and Tukey studentized range (HSD) test.

## 4.4 Results

### 4.4.1 MNPR1 gene cassette and Agrobacterium transformation

Due to the absence of suitable restriction sites within the original *MNPR1* sequences, cloning was facilitated via the insertion of additional sites at the 5' and 3' flanking regions. These restriction sites were chosen due to their unique presence in the MCS of plasmid *pBin19-LBR* (Fig. 4.1), as well as their absence in plasmid *pMNPR1A* and *pMNPR1B* (Fig. 4.2). Insertion of these restriction sites into the *Bam* *HV*/*Sma* I sites of *pGem-T-easy* was first confirmed after restriction enzyme digestion using a *Bam* *HV*/*Sma* I digestion (Fig. 4.4). Sequence analysis of the 1.8 kb *MNPR1* fragments revealed the successful addition of *Sma* I, *Xba* I and *Bam* HI sites in their correct orientation and position (Fig. 4.5).



**Figure 4.4** Restriction digest of *pGem:MNPR1A* and *pGem:MNPR1B* clones showing the 1.8 kb *MNPR1* fragment and the 3 kb *pGem-T-Easy* plasmid backbone. The presence of an upper 3 kb fragment (backbone of *pGem-T-easy vector*) and a lower 1.8 kb fragment (*MNPR1* inserts) were used as an indication for successful cloning.

## >*MNPRIA* (1769 bp)

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TAGGGATCCATATGGAAGACAACATCTCTACTGCGCCCCCGCGTTCTCCGTCCTCCGACAACAGCCGAGCGTCCACTTCGCCGGC
GGCGCATCTCCCGACCCCCTGCGGATGTGGAGGCCCTCCGCCGTTATCTGACAACCTTGGTGCCGATTTCGAGTCGCCGGACTT
CGAGTTGTTTCGCCGACGCCCGCATCGCTGTCGAGGACGGCGGGGCGCCGGCGCGGAGTTCGAGTCCACCGGTGCGTCTGTCCG
CGCGGAGCCCTTCTTTCGGGAGGTGTTTCGCGGAGCGGGAGGGGGCGCTGGCGCCGGTGGAGCTGTGGAAAGCTGGTGGAGC
GGTTCGCGGTTCGCTACGACGCCCTTGGTGACGGTCTCGGGTACCTTACAGAGGGAGGGTGGCGCCGCTGACCAAGGAGGTGTG
CATGTGCGTGGACGAAGAGTGCCTGGCAGGCGTGCCTGGCGCGGTGGTTCGATTTCATGGTTCGAAGTCTACGCCTCCTTCGCTC
TCCAAATCTCCGAGCTGGTCAGCCCTTCCAGCGACACCTTCTTGATATCTTTCGACAAGGTGTCTATAGATGACATTCGGTAATC
CTATCTGTGCTAACCTGTGTGATAGCTCTTGTGCCAAATATTTCAACAAGTGCATAGAGATTGCAGTTAAGTCAGACCTGGACAT
CATTACGCTTGAGAAGACAATGACCTCTGATATTTGTCAGCAAAATCATGGATTTCACGCTTGAATTTGGGAACAGTAGGACCTGAAA
GCATCAATTTTCTGATAAACATGTCAAGAGAATACATGGGGCTCTCGATAATGATGATGTTGATTTAGTAAGAATGCTGTTGAAAG
GAGGGGAATACAACACTAGATGATGCATGTCTTTCATTATGCAAGTGGCATAATTTGACTCAAAAGTTACAACAGAGCTATTAGA
TCTTGAACCTGCAGATATTAACCATAGAAACATCAGAGGCTATACAGTGTTCACATTGCTGCAATGCGTAAGGAAACCAAGATCA
TTGTGTCGCTACTAACAAGAGGAGCTAGACCTTCTGATCTAACACTAGATGGAAGGAAAGCAGTTTCAGATCTCAAGAGACACACC
AAGTCCATGGAGTACTTCAAGTCAACTGAAGAAGGACAAGCATCTCCCAAGAGTCGTTTGTGCATCGAGATATTAGAGCAAGCTGA
AAGAAGAGATCCCGAAGTAGGAGAAGCTTTCGCTTTCGCAATAGCTGGTGTGATGACTTGCAGGGGAAGATTACATATACCTTGA
ATCGAGTCACTCTTCAAGACTATTTATCCCAATGGAGGCAAGGGTTGCCATGGACATGCTCGAGTTGATGGCACATTAGAATTT
ACCTTAGGTTCTGCTACTAGCCATCTACGAAATCAAGAACTCAGCAGATCTAAATGAAACACCTTACAGATCAAGAGACA
GCATCTAGCACGTATGAGAGCACTTCCAGAACAGTGGAACTCGGGAAGCGCTTTTTTCCCGGTGCTCAGCGGTTATCAATAAGA
TCATGGATGATGACCCGACAGATTTGCTTATCTTCAGCATGATGCATCAGAAGAGAAGGATGAGATTTTTGGAACGTCAGGAT
GCCCTGTGAGAGCATTACGCGAGGACAAGGAGGAATTCACAAGTCTGCTTTGTCTTCTCATCATCAACATCGGTCCGCAT
TGTACCAACACAAAGATGATACATTTGTCGCTGATCCCGGGTCTAGACTA

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## >*MNPRIB* (1839 bp)

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TAGGGATCCACACGGCGGGACATGGAACCCAGCTACCTCACGGCCGCCACCGCCTTCTCGGGCTCCGACAACAGCAGCTGCGTGCA
CTTCTCCGGCGATGCGGCGGGCTGCTGCAGCTCCGGACTCCGCCCGCCCGCGGGGAGGTCGAGGGACTCCGTCGCCTGTCCGACC
ACCTCGGCTCCGCCCTCCAGTCCGCGGACTTCGAGTTCTCTCCCGACCGCCCGCATCCGCGTCCGGCCCCAGGGGACCGCGGGTCA
ACGCCCGCGGAGGTTCGCGGTACACCGCTGCGTGCTATCCGCCCGGAGCATCGTATCCGGGAGGAGTTCGCGAGGGCGGGGAGGGG
AACGGCCCGCGGCCCGGTGAGGATGGAGTGAAGGAGCTGTTAAAGGACTTCGAGGTCGGGTACGACGCTTGGTGGCGGTGCTCG
GGTACCTCTACACCGGGAGGGTGGCACCCTGCCCCAAGCGGTGTGCGCTGCGTTCGACGAGGAGTGCCTGGCACGAGGCGTCCCGG
CCGGCGGTGATTTTCATGGCCGAAGTGTCTACGCCCTCCTCCGCTTTCCAAATCCCGAGCTGGTTCAGCTCTCCAGCGGCACCT
CCTTGGTATCTGGACAAGATGGCAATAGATGACATACCAGTAATTTCTCTGTTGCTAAACTATGCGATAGCTCATGCGCCAATC
TGCTCAGCAAATGCATAGACGTTGTAGTCAAGTCAAGTCAAGTCAAGTCAAGTCAAGTCAAGTCAAGTCAAGTCAAGTCAAGTCAAG
CAAATATGGATTTACGCTTGAATTTGGGCTAGTGGGACCTGAAAGCAGCAGCTTTCCTGATAAACACGTCAAGAGAATACATAG
AGCTCTTGACAGTATGATGTTGACTTAGTAAGAAATGCTATTAAGAGGAGGGAATACAACGCTAGATGACGATGTGCATTTGATT
ATGCGGTAGCATATTTGATTTCAAAAATCAACAACAGAGCTGTTAGATCTTGCCTGGCAGATGTTAACCATAGAGACTTCAGAGGT
TATACTGTGCTTCACATAGCTGCAATGCGTAAAGAACCCTAAGATCATCGTGTCACTTCGACAAGGGAGCCAGACCATCTGATCT
TACATTTGATGGAAGGAAAGCACTTCAGATTGCAAAAGAGACTTACCAAGTCTGTGGAGTACCTCAGGTCGATTGAAGAGGAGAAG
CATCTCCTAAGAGTCTGTTGTGCTATTGAGATATTAGAGCAGGCTGAAAGAAGAGATCCACAAGTAGGTGAAGCTCTGTGATCACTT
GCAATGGCTGGTGTGACTTGCAGGGAAGATTGTTGATCTTGGAAATCGAGTTGCTTGGCAAGACTATTGTTCCCATGGAGGC
AAGAGTTGCTATGGACATTCGACAAGTTGATGGACATCGGAGTTCACCTTAGGGTCTACCAGCAACCGTTCTACTGGAAATCAA
GGACTGCGATGGATCAAAACGAAGCACCATTCAAGATCAAGGAAGAGCATCTGGCACGAAATGAGAGCACTTCCAGAACAGTGGAA
CTTGGGAAGCGCTTTTTCCCTCGGTGCTCAGAGGTCATCAACAAGATCATGGACGACGATCTCACAGAAATCACTGGCCCTCGGACA
CCACACTTCGGAGGAGAAGAGGAGGAGATTTAGGAGTTGCAGGAAGTCTGTCAAAAGCATTTAGCCAAAGACAAGGAGGAATTCG
ACAGGTCGCTTGTCTTCTCATCTCTCATCTGATCAACATCCATCGACAAGGTTTGGCCGAACAAGAAGATGAGATTCATTTCCCA
CCTAGTTCGATCTATACCCTCCGGTCTAGACTA

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**Figure 4.5** Full length *MNPRIA* and *MNPRIB* coding region with added restriction sites.

Primers (rectangular boxes) flanking the 5' and 3' regions of the *MNPR1* coding sequence were used for amplification of *pMNPRIA* and *pMNPRIB* plasmids. The start and stop codon (grey), *Bam* HI, *Sma* I and the *Xba* I (bold and italicized) were all identified after sequence analysis of selected clones.

Restriction enzyme digest of full length *MNPR1A* and *MNPR1B* coding sequence with *Bam**HI/Sma* I further demonstrated successful insertion of coding sequences into the plasmid *pBin19-LBR* to obtain plasmids *pLBR:MNPR1A* and *pLBR:MNPR1B*. Sequencing of the gene cassette in the binary vector showed that the respective banana *NPR1* sequences had been inserted in the correct orientation with the banana *NPR1* genes under the control of the 35S CaMV promoter and terminator sequence (Fig. 4.6). Plasmids were then used to transform competent LBA4404 *Agrobacterium* cells to obtain cells carrying *pLBA:LBR* (vector alone), *pLBA:MNPR1A* and *pLBA:MNPR1B* allowing *Agrobacterium*-mediated transformation of *npr1-2* mutant plants.

>pLBR-MNPRI A partial sequence information

```

-----
GTTCATTTTCATTTGGAGAGGACAGCCCAAGCTTGGCTGCAGGTCGAC
GGATCCATATGGAAGACA ACTATCTCACTG-----
-----
TTGTACCAACACAAAGATGATACAT TGTGCTGTATCCCGGGGAATTC
GGTACGCTGAAATCACCAGTCTCTCTCT-----

```

>pLBR-MNPRI B partial sequence information

```

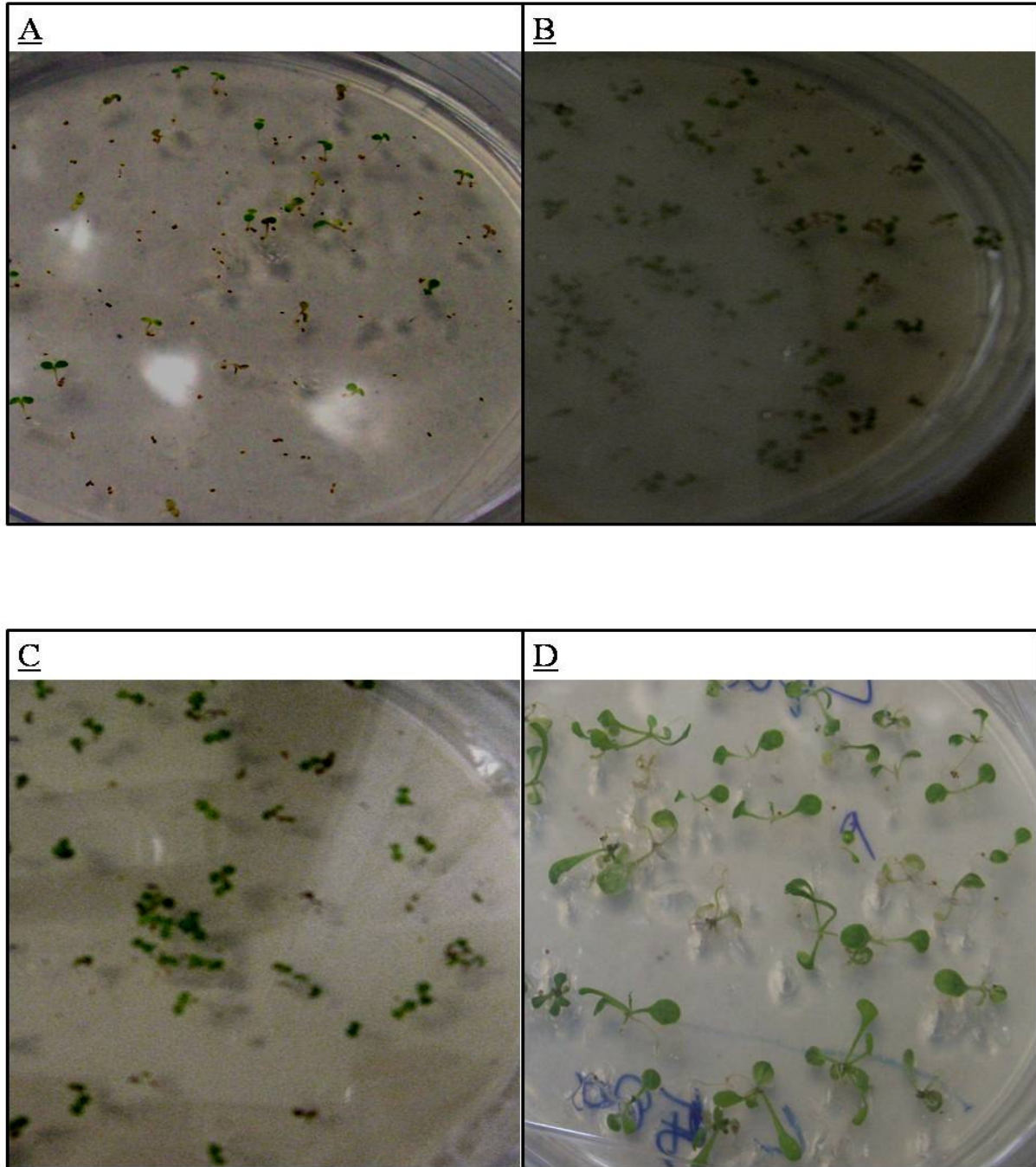
-----
TTCATTTGGAGAGGACAGCCCAAGCTTGGCTGCAGGTCGACGGATCC
ACACGGCGGGACATGGAACCCAGCTACCTCACGGCCGCCACC-----
-----
AGATGAGATGATTCCACCTAGTCGATTCTATAACCCCCGGGGAATTC
GGTACGCTGAAATCACCA-----

```

**Figure 4.6** Partial sequence of the 35S CaMV promoter: *MNPRI*:35S CaMV terminator gene cassette. Dashes (-----) represent sequence information not shown. Sequences in grey delineate the junction sites between the 35S promoter and 5' end of the *MNPRI* sequence; and the 3' end of the *MNPRI* sequence and the terminator sequence. The start and stop codon of the *MNPRI* sequences are underlined. Bold and italicized sequences represent sites at the 5' and 3' end of the *MNPRI* coding region.

#### 4.4.2 Transformation and selection of homozygous transgenic lines

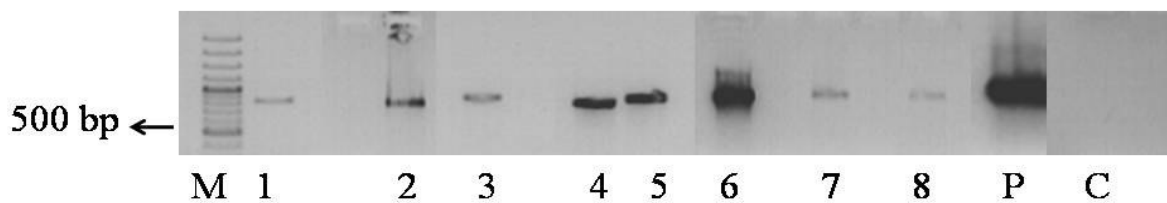
Using the floral dip method, *Arabidopsis npr1-2* mutant plants were successfully transformed with either plasmid p*Bin19:LBR*, p*LBR:MNPR1A*, and p*LBR:MNPR1B*. Selection of more than 1000 T-1 seeds from putative transformed plants with each of the constructs initially yielded a transgenic plant recovery rate of about 16-20% (Fig. 4.7). However, following further screening via PCR, only between 16-18% of these regenerants had the gene integrated within their genome (Fig. 4.8) and these were considered as “true” transformants. During the second selection step (T-2 selection) of seeds, about 50% of seeds regenerated on kanamycin. During the T-3 selection, more than 65% of seeds survived on the selection medium and at the T-4 selection stage more than 95% of positive transformants were obtained which were used for further analysis (Fig 4.8).



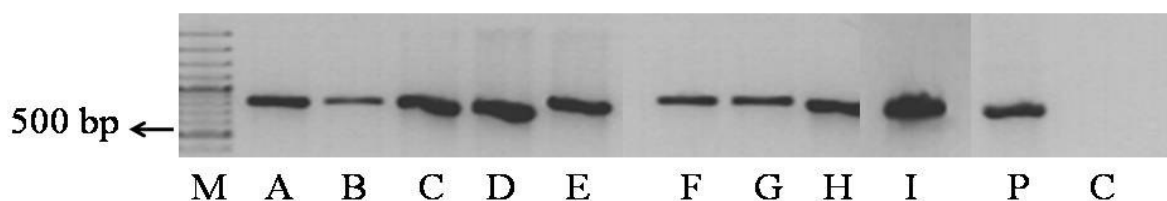
**Figure 4.7** Kanamycin selection of plants derived from an *npr1* transgenic event. Following floral dipping of the *Arabidopsis npr1* mutant with *pLBA:LBR*, *pLBA:MNPR1A* and *pLBA:MNPR1B* inoculum, T-1 seeds produced were selected on kanamycin-containing MS agar

plates. Selection process was successively repeated over 4 generations (T-1 to T-4 selection) of self fertilization.

*npr1:MNPR1A* (T-1)



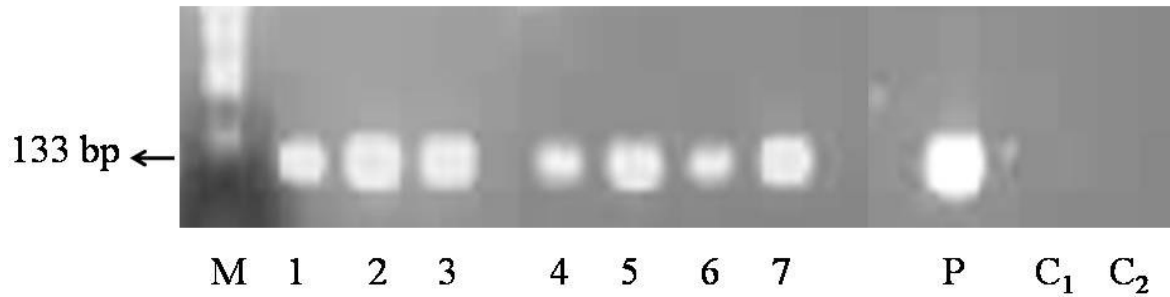
*npr1:MNPR1B* (T-1)



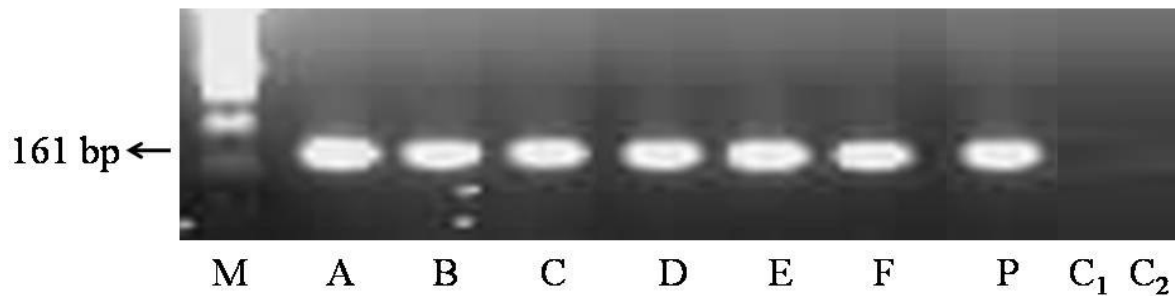
**Figure 4.8** 800-bp PCR products from putative transformed *Arabidopsis* plants (T-1 generation) carrying *npr1:MNPR1A* and *npr1:MNPR1B* DNAs after kanamycin selection. Numbers (1-8) represent the various independent transformation with *MNPR1A* while letters (A-I) are with the *MNPR1B* gene. The controls used during the PCR were as follows: P = plasmid (positive) control; C = *npr1* DNA (negative) control. M represents a DNA marker to determine fragment size.

Further confirmation of stable gene integration using homozygous lines obtained after four successive kanamycin selection stages showed that the *MNPR1* coding sequence could be successfully amplified from cDNA (Fig. 4.9).

*npr1:MNPR1A* (homozygous lines)



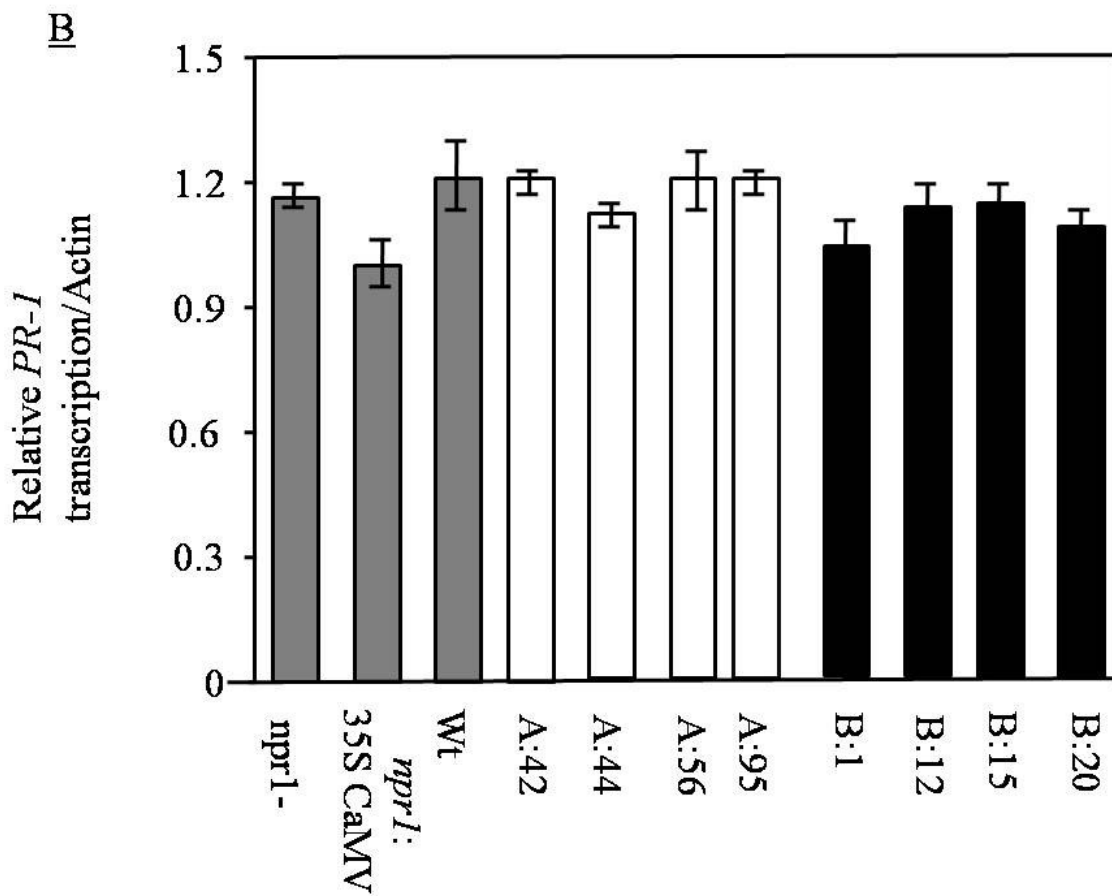
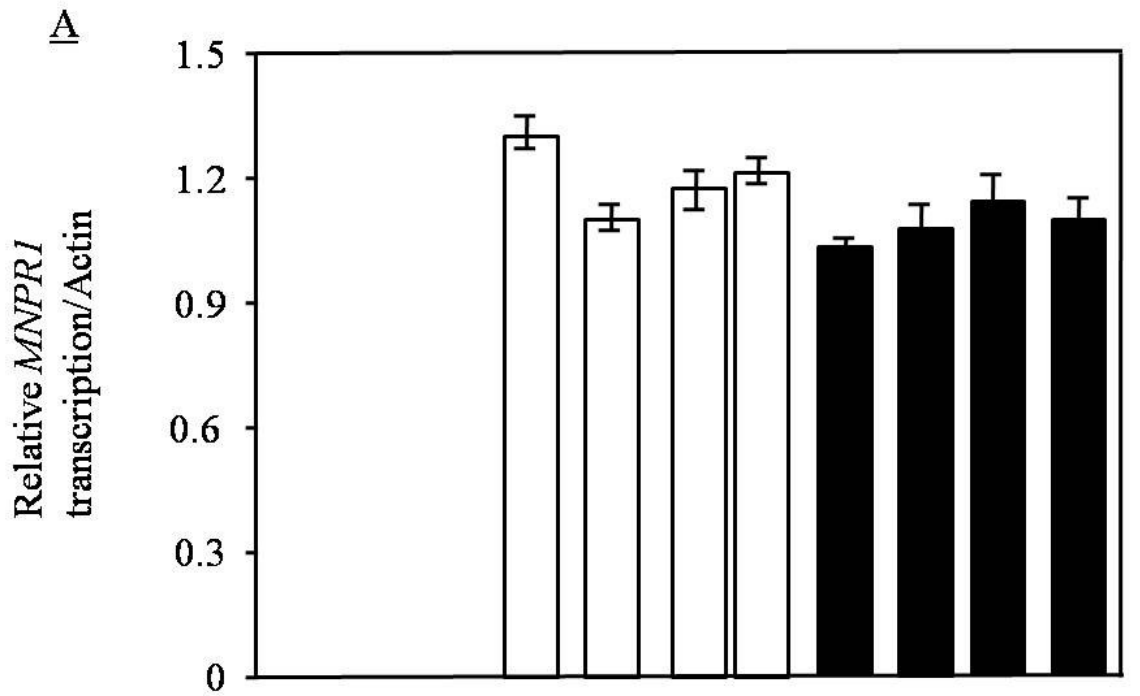
*npr1:MNPR1B* (homozygous lines)



**Figure 4.9** PCR products from homozygous *npr1:MNPR1A* and *npr1:MNPR1B* plants. PCR was conducted using cDNA of homozygous *npr1:MNPR1A* and *npr1:MNPR1B* transgenic plants. Numbers on gel represent *npr1:MNPR1A10*, *npr1:MNPR1A42*, *npr1:MNPR1A44*, *npr1:MNPR1A56*, *npr1:MNPR1A95*, *npr1:MNPR1A204* and *npr1:MNPR1A312*. Letters on gel represent lines: *npr1:MNPR1B1*, *npr1:MNPR1B8*, *npr1:MNPR1B9*, *npr1:MNPR1B12*, *npr1:MNPR1B15* and *npr1:MNPR1B18*. M represents the DNA marker for determination of fragment size. Controls were as follows: P = plasmid (positive), C<sub>1</sub> = *npr1* cDNA (negative) control, C<sub>2</sub> = wild-type cDNA (negative).

#### 4.4.3 *MNPR1* and *PR-1* gene transcription

Quantification of both the *MNPR1* and *PR-1* relative to the endogenous *Arabidopsis* actin gene transcript revealed a 1- to 1.2-fold *MNPR1A* and *MNPR1B* higher transcript amount for four of the selected *npr1:MNPR1A* and *npr1:MNPR1B* transgenic plants tested (Fig. 4.10A). There was no significant differences in both *MNPR1* and *PR-1* transcript levels between the different transgenic lines ( $p>0.05$ ). No *MNPR1A* or *MNPR1B* transcripts were detected in the various control plants (Wt, *npr1* and *npr1:35S CAMV Arabidopsis* plants). However, a 1- to 1.4-fold higher *PR-1* gene transcript level was detected in all the controls as well as the various *npr1:MNPR1A* and *npr1:MNPR1B* transgenic plants tested (Fig. 4.10B).



**Figure 4.10** *MNPR1* and *PR-1* transcript amounts in tested *Arabidopsis* plants. *MNPR1A*, *MNPR1B* (A) and *PR-1* (B) transcription was measured in various *Arabidopsis* genotypes using cDNA from 5 week-old plants. The quantity of transcript was expressed relative to the endogenous actin transcript. The mean transcription values  $\pm$  the SEM from two independent samples are shown. Samples represented consist of: controls (grey bars; non-transgenic *npr1*, *npr1* transformed with the 35S CaMV promoter/terminator sequence and non-transformed wild type), *npr1:MNPR1A* lines (white bars; consisting of line A-42, A-44, A56, A95) and *npr1-MNPR1B* lines (dark bars; B-1, B12, B15, B20).

## 4.5 Discussion

This is the first report on the expression of banana *NPR1* genes in *Arabidopsis npr1-2* mutant plants. The model plant *Arabidopsis* was used due to the lack of an established and routine transformation system for banana at the beginning of this study. *Arabidopsis* was a suitable surrogate to study gene function as the *npr1* genes have been studied extensively in this species and, unlike banana, mutants in the *npr1* gene that are suitable for validation of gene function are available. Previous research has shown that a native *NPR1* gene can be stably integrated into *Arabidopsis npr1* mutant plants by using a homologous *NPR1* gene, or through back crossing of mutant *npr1* plants with wild-type *Arabidopsis* plants (Cao *et al.*, 1994; Yuan *et al.*, 2007; Sandhu *et al.*, 2009).

In this study, the dipping method for *Arabidopsis* transformation produced a large number of non-transgenic “escape” plants. Although the floral dip method is well established and routinely used in *Arabidopsis* transformation, an adequate selection of F1 progenies and homozygous lines is continuously being optimized so as to obtain higher regeneration efficiencies (Clough and Bent, 1998; Xiang *et al.*, 1999; Harrison *et al.*, 2006; Dehestani *et al.*, 2009). Harrison *et al.* (2006) reported by screening of about 3200 seeds derived from transgenic plants obtained by the floral dipping method indicates that out of 118 regenerated plants, 110 had detectable expression of the candidate gene. In this study, more than 50% of plants derived from the transformation process were not transformed. Such low frequency might be due to (i) the large number of seeds plated per 90-mm Petri dish in this study where too many seeds on the plate might have promoted detoxification of the antibiotic and hence favor the regeneration of non-transformed

seeds (Wilmink and Dons, 1993), (ii) the number of days the seeds were selected on kanamycin which might have been too long and might have allowed degradation of the antibiotic (Harrison *et al.*, 2006; Dehestani *et al.*, 2009) and (iii) seeds might have only transiently expressed the transgene with no expression of the transgene after potting the plants thus accounting for non-detection of the transgene in the plant genome by PCR (De Buck *et al.*, 1998). However, the transgenic plants recovered were deemed adequate for the work planned in this study.

In this study, it was important to elucidate if the transformation process or the presence of the *MNPR1* genes did not result in an alteration of *PR* gene expression in the absence of pathogenic or other perturbation. In homozygous lines expressing banana *NPR1*, *Arabidopsis PR-1* transcripts were not affected by *NPR1* expression. This was found by application of qRT-PCR technique which is currently the most sensitive technique to determine amounts of transcripts (Sandhu *et al.*, 2009, Divi *et al.*, 2010). Further, results found in this study also confirm previous findings by Kinkema *et al.* (2000) that expression of *NPR1* genes does not activate *PR-1* transcription under non-stress conditions.

In the next chapter expression of *MNPR1A* and *MNPR1B* was investigated in addition to expression of the *Arabidopsis PR-1* gene after infection with distinct classes of pathogens.