Two different banana *NPR1*-like coding sequences confer similar protection against pathogens in *Arabidopsis*

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Abstract

Differences between the coding sequences of two banana homologues of the *Non-expressor of Pathogenesis-Related 1* (*NPR1*) genes (*MNPR1A* and *MNPR1B*) were investigated as a possible cause for the differential activity of the two genes. Each of the *MNPR1* coding sequences were expressed under the control of the cauliflower mosaic virus 35S promoter/terminator sequences in the transgenic *Arabidopsis npr1-2* mutant. These *MNPR1*-expressing plants were then exposed to either the biotrophic oomycete *Hyaloperonospora arabidopsidis*, the necrotrophic fungus *Botrytis cinerea*, or the hemi-biotrophic bacterial pathogen *Pseudomonas syringae*. Expression of either *MNPR1A* or *MNPR1B* increased

Pathogenesis-related 1 (*PR-1*) transcription in a similar manner in mutant plants and reduced pathogen growth, restoring resistance of the *Arabidopsis npr1-2* mutant plants to pathogens. Sequence differences between *MNPR1A* and *MNPR1B* coding sequences did not affect *MNPR1*activity, suggesting a possible role for the involvement of other regulatory sequences in differential *MNPR1* gene expression.

Keywords: Banana *NPR1*-like sequences; *Botrytis cinerea*; *Hyaloperonospora arabidopsidis*; PR proteins; *Pseudomonas syringae*; sequence dissimilarity.

Introduction

The *Non-expressor of Pathogenesis-Related 1* (*NPR1*) gene is a defence response co-transcriptor conferring resistance to a wide range of pathogens during the establishment of systemic acquired resistance (SAR) and induced systemic resistance (Pieterse and van Loon 2004; Wally *et al*., 2009). This is due to the ability of NPR1 to activate various forms of antimicrobial *Pathogenesis-Related* (*PR*) genes (Cao *et al*., 1994; Glazebrook *et al*., 1996; Cao *et al*., 1997; Yuan *et al*., 2007; Canet *et al*., 2010; Shi *et al*., 2010).

Whole-plant genome sequencing has revealed the existence of a *NPR1*-like gene family with the proteins they encode sharing typical features that are important for their function, such as ankyrin repeats and bric-a-brac/poxviruses and zinc-finger (BTB/POZ) domains (Cao *et al*., 1997; Argout *et al*., 2011). In *Arabidopsis thaliana*, in addition to the *NPR1* gene, 5 homologous *NPR1*-like sequences have been identified and characterized (*Arabidopsis* genome initiative, 2000). Further, *Oryza sativa* (rice), *Medicago trunculata*, *Poplar trichocarpa* (poplar), *Vitis vinifera* (grape), and *Theobroma cocoa* (cocoa) harbour 5, 4, 6, 3 and 4 *NPR1*-like sequences, respectively (Liu *et al*., 2005; Yuan *et al*., 2007; Argout *et al*.,

2011). Recent phylogenetic analysis has also placed *NPR1*-like genes into three distinct clades (Bergeault *et al*., 2010; Argout *et al*., 2011). Sequences belonging to clade 1, including *Arabidopsis NPR1* and *MNPR1*, act as positive mediators of the pathogen defence response (Cao *et al*., 1997; Bergeault *et al*., 2010; Argout *et al*., 2011).

In banana, genome walking and rapid amplification of cDNA ends (RACE) have allowed the isolation of three *NPR1*-like sequences: two from Cavendish banana AAA (*MNPR1A* and *MNPR1B;* Endah *et al*., 2008), and one from ABB Dongguan dajiao *Musa* spp. (*MdNPR1*; Zhao *et al*., 2009). Characterization of these banana *NPR1*-like genes has been limited to transcription analysis without any direct proof of activity. Salicylic acid (SA) treatment, or infection with *Fusarium oxysporum* forma specialis (f. sp.) *cubense* race 4 (*Foc*), resulted in increased amounts of *MdNPR1*transcripts in the *Fusarium*-resistant cultivar Dongguan Dajiao when compared to the *Fusarium*-sensitive cultivar Fenjiao (Zhao *et al.*, 2008). Endah *et al*. (2008) measured *MNPR1A* and *MNPR1B* transcription in a *Foc*-sensitive (Grand Naine; Stover and Buddenhagen, 1986), and a *Foc*-tolerant (GCTCV-218; Hwang and Ko, 2004) banana cultivar after *Foc*, SA, or MeJA treatment. In a previous study (Endah *et al*, 2008), *Fusarium*-treated *Foc*-tolerant GCTCV-218 plants displayed more and earlier transcription of both *MNPR1* genes was associated with greater accumulation of transcripts of *PR-1* and *PR-3* compared to *Foc*-sensitive Grand Naine plants. In addition, differential activation of the two *MNPR1* genes to the necrotrophic pathogen (*Foc*) also occurred. *MNPR1A* was more responsive to *Foc* treatment and *MNPR1B* more responsive to SA treatment. Both genes were, however, responsive to treatment with MeJA, and the response was cultivar dependent. Significantly more *MNPR1A* transcripts were in the *Foc*-tolerant cultivar than in the susceptible cultivar. The response of *MNPR1B* was transient in the *Foc*-tolerant cultivar and a more gradual increase *MNPR1B* transcription occurred in the *Foc*-sensitive cultivar (Endah *et al*., 2008). However, comparison of individual MNPR1A and MNPR1B activity in providing

pathogen resistance and investigation of the effect of coding sequence dissimilarity between the two *MNPR1* sequences have so far not been carried out.

A mutant complementation approach was therefore applied in our study with the aim of determining the contribution of each of the MNPR1s to the induction of *PR-1* transcription and of pathogen resistance in the *Arabidopsis npr1-2* mutant lacking *NPR1* activity. Further, we examined whether the difference between the *MNPR1* coding sequences causes any differential activity of the two MNPR1s. Homozygous transgenic *Arabidopsis npr1-2* mutant plants expressing the two *MNPR1* coding regions individually under the control of the constitutive 35S CaMV promoter/terminator sequences were infected with the biotrophic oomycete *H. arabidopsidis*; necrotrophic fungus *B. cinerea*; or hemi-biotrophic *P. syringae*. Complementation of *Arabidopsis npr1* mutants with *NPR1* genes has been shown to activate *PR* genes, restoring the pathogen-resistant phenotype (Yuan *et al*., 2007; Sandhu *et al*., 2009; Shi *et al*., 2010). Results of our study show that both *MNPR1*s were equally active in *Arabidopsis* mutants in inducing *PR-1* transcription and in restoring the pathogen-resistant phenotype and that differences in sequence between the two *MNPR1* sequences had no role in the degree of protection.

Results

We compared the ability of two *MNPR1* coding sequences with a 78% codon identity to elicit protective responses to pathogens using a transgenic complimentary approach. Three independent *npr1-2* homozygous transgenic lines expressing either *MNPR1*A (*npr1*:M*NPR1*A44, *npr1*:*MNPR1A56*, *npr1*:*MNPR1A95*), or *MNPR1*B (*npr1*:*MNPR1B1*, *npr1*:M*NPR1*B12, *npr1*:*MNPR1B15*) coding sequences under the contol of the 35S CaMV

promoter/terminator sequence as well as transgenic *npr1-2* plants carrying the empty vector (*npr1*:35S CaMV), and wild-type *Arabidopsis* Col-0 plants (Wt) were used for this study.

PR-1 transcription

Expression of *MNPR1* was confirmed by PCR amplification of cDNA synthesised from each transgenic line using gene specific primers *MNPR1* was detected in all *MNPR1*-transgenic-lines while no amplification products were obtained from the wild-type plants and *npr1:35SCaMV Arabidopsis* plants using these *MNPR1*-specific primers (data not shown). The basal *PR-1* transcription in the *MNPR1*-expressing lines was also determined and was not significantly different ($P>0.05$) from that of the wild-type and *npr1:35SCaMV Arabidopsis* control plants (data not shown).

Following infection with *H. arabidopsidis*, *PR-1* transcription significantly ($P<0.05$) increased by up to 47-fold in all the *MNPR1*-expressing lines when compared to non-infected transgenic mutant plants or mutant plants only carrying the 35S CaMV promoter/terminator sequence (Fig. 1A). *PR-1* transcription in the individual *MNPR1*-expressing plants was not significantly ($P>0.05$) different from each other and was furthermore similar to *PR-1* transcription in wild-type non-transformed *Arabidopsis* plants (Fig. 1A).

After infecting all categories of plants under study with *B. cinerea*, *PR-1* transcription was significantly ($P<0.05$) increased, up to 50-fold in plants of all *MNPR1*-expressing lines. Except for *npr1*:*MNPR1B1*,which had a smaller increase (22.8-fold) relative to non-infected plants or *npr1*:35S*CaMV* control plants, not significant differences ($P>0.05$) in *PR-1* transcript was observed between the *MNPR1A*- and *MNPR1B*-expressing lines (Fig. 1B). Amounts of *PR-1* transcripts measured in these *MNPR1*-expressing mutant plants were also

not significantly (P>0.05) different from those in wild-type *Arabidopsis* plants after *B. cinerea* infection (Fig. 1B).

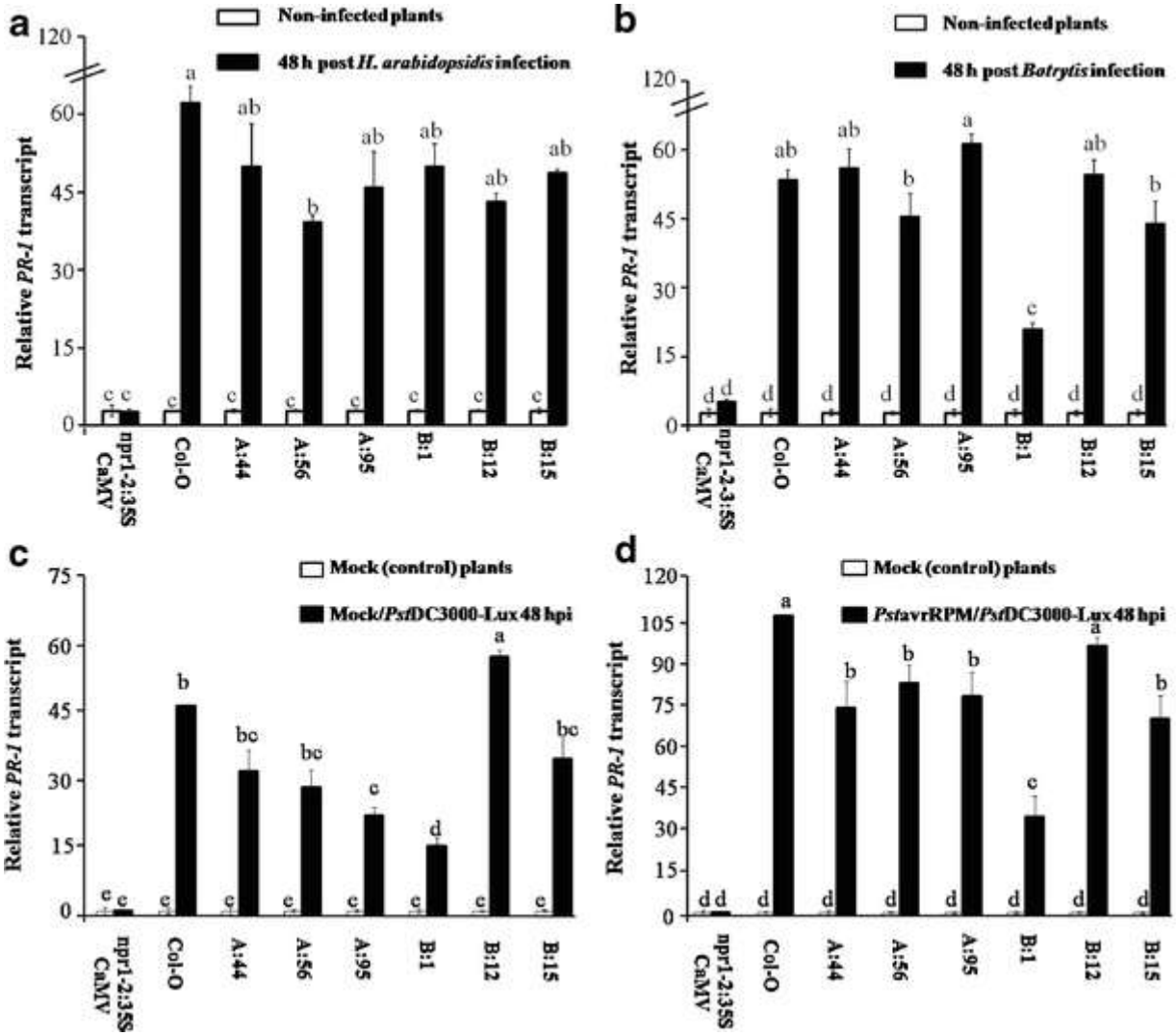

Figure 1 Relative *PR-1* transcript amounts in various *Arabidopsis* genotypes after pathogen infection. Various *Arabidopsis* genotypes were treated with either the biotrophic pathogen *H. arabidopsidis* isolate NOCO2 (A); or the necrotrophic fungus *B. cinerea* (B); or the hemi-biotrophic bacteria *Pst*avrRPM1 and *Pst*DC3000-Lux (C, D). Control plants were treated in the same manner using the re-suspension buffer for each pathogen. *PR-1* transcript amounts were measured using cDNA from leaf samples harvested from the infected and control plants 48 hpi. Values obtained were normalized with values from their endogenous control gene (ACTIN2) and *PR-1* transcripts expressed relative to the control. The experiment was repeated once and the mean relative transcription values ± SEM of both experiments was plotted. The significant difference among samples was determined using SAS$^{(R)}$ software. Bars on the same graph with unidentical letters (a, b, c, d) are significantly different (P<0.05).

PR-1 transcription was measured in the various plant types after SAR-induction (AV-; *Pst*avrRPM1/ *Pst*DC3000-Lux-treated plants) and non-SAR-induction (MV-; mock/*Pst*DC3000-Lux-treated plants). In the MV- (mock/*Pst*DC3000-Lux)-treated plants, *PR-1* transcription increased significantly ($P<0.05$), up to 57-fold in the *MNPR1*-expressing lines in comparison to *PR-1* transcription in the MV-treated *npr1*:*35SCaMV* control plants (Fig. 1C). But for *MNPR1B*:1 and *MNPR1B*:12, these *PR-1* transcripts were not significantly different ($P>0.05$) from each other or from *PR-1* transcription of the MV-treated wild-type plants.

Similarly, following SAR-induction a significant increase ($P>0.05$) in *PR-1* transcription of at least 34-fold was observed in all *MNPR1*-expressing lines compared to *PR-1* transcription in the SAR-induced *npr1*:*35S* CaMV control plants (Fig. 1D). Among these SAR-induced *MNPR1*-expressing lines, only *MNPR1B*:12 had *PR-1* transcripts (96-fold) similar to *PR-1* transcription in the SAR-induced wild-type plants (107-fold). Also, *MNPR1B*:1 had the least *PR-1* transcription (34-fold) among the various SAR-induced *MNPR1*-expressing lines and this was significantly smaller ($P<0.05$) than *PR-1* transcription in the other SAR-induced *MNPR1*-expressing lines.

<u>*MNPR1*-induced protection against pathogens</u>

In a next step we determined the presence and number of conidiophores after plant infection with the biotrophic fungus *H. arabidopsidis* by light microscopy and after trypan-blue staining of entire, infected *Arabidopsis* rosettes. The *H. arabidopsidis* count was significantly ($P<0.05$) lower 7 dpi in *MNPR1A*-expressing plants (68% reduction) and in *MNPR1B*-expressing lines (up to 73% reduction) as well as wild-type plants (53% reduction) compared

to *npr1*:*35SCaMV* control plants. However, there was no significant difference (P>0.05) in counts between *MNPR1A* and *MNPR1B* lines (Fig. 2).

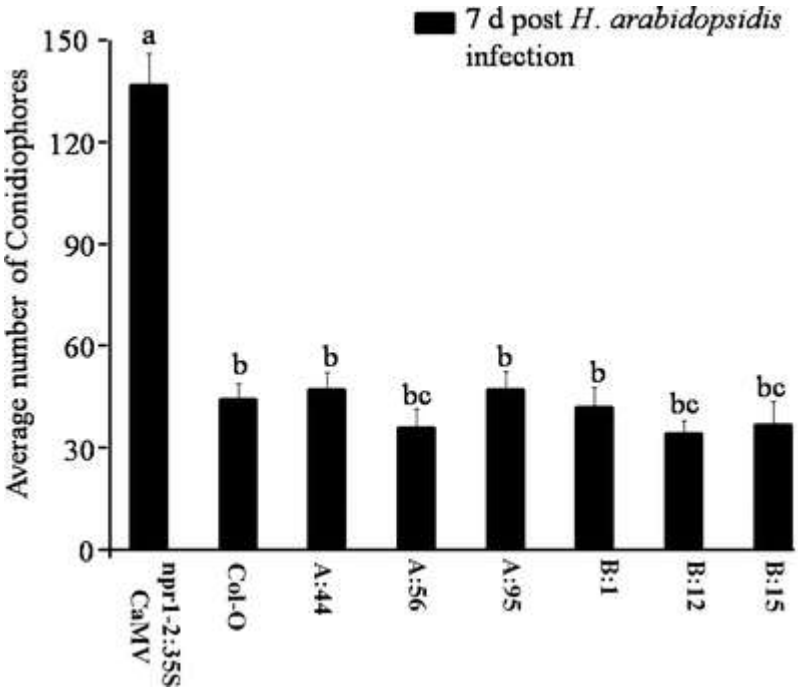

Figure 2 *H. arabidopsidis* conidiophores present in various *Arabidopsis* genotypes 7 days after infection. *Arabidopsis* genotypes (eight) were sprayed with a 2.5 x 10^4 spores/mL of a *H. arabidopsidis* inoculum. The mean conidiophores number obtained 7 dpi from 12 plants per genotype was plotted together with the SEM. The significant difference among samples (represented by the unidentical letters a, b, c, on each bar) was determined using SAS$^{(R)}$ software. The experiment was repeated twice using independent sets of plant material treated in the same manner described above.

We also measured the lesion diameters on leaves infected with *B. cinerea*. All *MNPR1*-expressing mutant plants had significantly (P<0.05) lower lesion diameters at 72 hpi ranging from 38% to 47%, and 32% to 35% in the *MNPR1A* and *MNPR1B* expressing lines respectively, when compared to *npr1*:*35S* CaMV control plants (Fig. 3). However, there was no significant (P>0.05) reduction in lesion diameter between *MNPRA* and *MNPR1B* lines. Infection of leaves of *npr1*:*35S* CaMV control plants with *B. cinerea* also resulted in brownish-soaked lesions spreading from the infection site (beyond 6 mm) with further degeneration into yellowish secondary necrotic lesions as the pathogen continued to invade

(Fig. S1; supplementary material). However, in *MNPR1*-expressing plants, these lesions were limited to the infection site, not spreading beyond 4 mm on the leaf surface during the 72 h infection period.

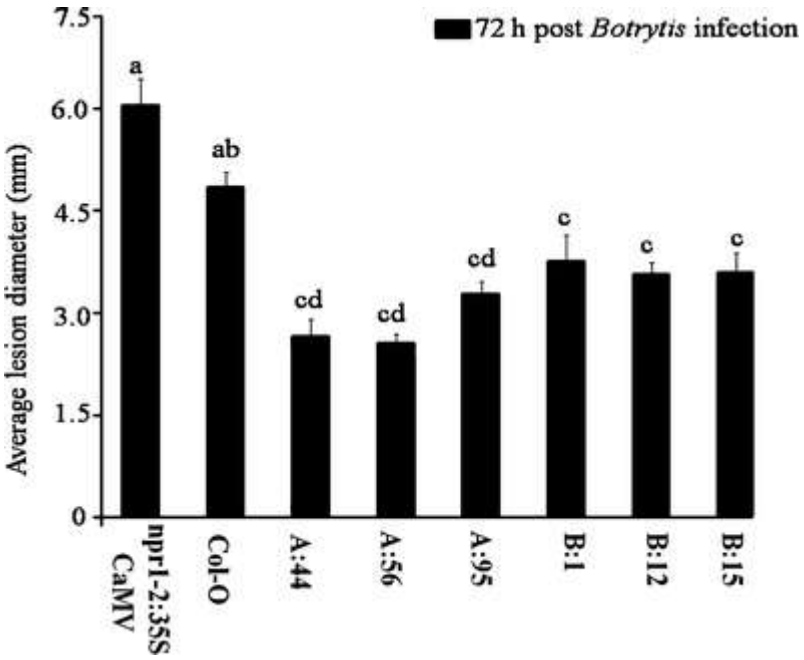

Figure 3 Lesions diameter on leaves of various *Arabidopsis* genotypes 72 hpi with the necrotrophic fungus *B. cinerea* B05.10. Leaves (six per plant) of eight different *Arabidopsis* genotypes were infected with a 2.5 x 10^5 spores/mL of an inoculum of *B. cinerea* B05.10. The mean lesion diameter from 36 leaves per genotype was measured 72 hpi and plotted together with their SEM. The significant difference among samples was determined using SAS(R) software and samples which were significantly different from each other were represented by unidentical letters (a, b, c, d) on the graph. The experiment was repeated twice using independent sets of plant material treated in the same manner described above.

Next we measured *Pst*-DC3000-Lux bacterial growth 48 hpi in non-SAR-induced and SAR-induced plants using a very sensitive quantitative bioluminescence assay. This facilitates *in planta* quantification of bioluminescent *Pst*-DC3000-Lux bacteria due to the presence of the LuxCDABE operon from *Photorhabdus luminescens* inserted into the *P. syringae* chromosome (Fan *et al*., 2008). During the non-SAR induced treatment (mock/*Pst*DC3000-Lux-treatment), growth of *P. syringae* was significantly ($P<0.05$) lower, at least 53% (*MNPR1A*) and 50% (*MNPR1B*) in comparison to the *Arabidopsis npr1*:*35S* CaMV control

plants (Fig. 4A). This reduced pathogen growth found in the *MNPR1*-expressing lines was not significantly (P>0.05) different between the various complemented lines or from that of infected wild-type *Arabidopsis* (61%).

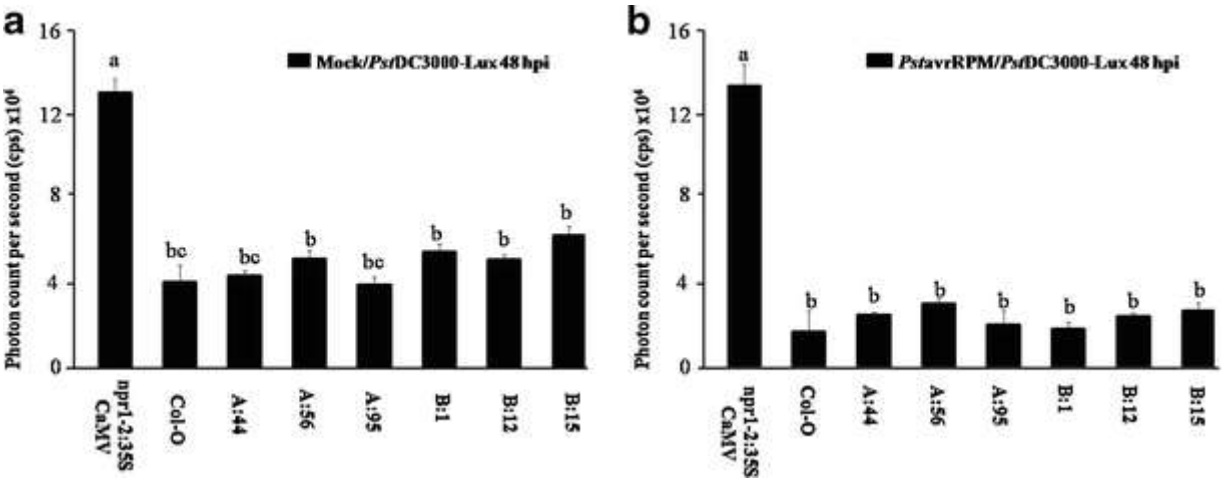

Figure 4 Growth of the hemi-biotrophic *Pst*DC3000-luxCDABE (*Pst*DC3000-Lux) in various *Arabidopsis* genotypes before and after SAR induction. *Pst*-DC3000-Lux bacteria growth was measured 48 hpi in non SAR-induced (A; Mock-*Pst*-DC3000-Lux treated) and SAR-induced plants (B; *Pst*avrRPM1-*Pst*-DC3000-Lux treated) plants using a FB12 luminometer. The mean bacteria growth from 36 leaves per genotype was plotted together with their SEM. The significant differences between samples was determined using SAS$^{(R)}$ software and bars with unidentical letters (a, b, c, d) were considered to be significantly different (P<0.05).

Similarly, after SAR-induction (*Pst*avrRPM/ *Pst*DC3000-Lux-treatment), a significant reduction (P<0.05) in *Pst*DC3000-Lux proliferation of at least 73% and 76% was obtained for the *MNPR1A*- and *MNPR1B*-expressing lines respectively, in comparison to the SAR-induced *Arabidopsis npr1*:*35S* CaMV control plants (Fig 4B). Reduction in *Pst*DC3000-Lux proliferation between the *MNPR1*-expressing lines was not significantly different (P>0.05) from the wild-type (89%) plants. Furthermore, *npr1*:*35SCaMV* leaves were more chlorotic with more spreading lesions when compared either to wild-type plants or *MNPR1*-expressing plants after SAR-induction and non-SAR-induction (Fig. S2; supplementary material).

Discussion

Our study has extended the group of already identified *NPR1* sequences from crops, such as soybean, cocoa, and rice, that are capable of restoring pathogen resistance by complementation of *Arabidopsis npr1* mutants (Yuan *et al*., 2007; Sandhu *et al*., 2009; Shi *et al*., 2010). In our study, both *MNPR1* coding sequences (*MNPR1A* and *MNPR1B)* complemented pathogen-sensitive *Arabidopsis npr1-2* mutants, activated downstream *PR-1* transcription and restored pathogen-resistance to wild-type levels. They therefore appear to represent two true banana *NPR1* orthologous sequences with similar functions as positive defence regulators in banana.

Our study also investigated the effect of sequence differences in two banana *MNPR1* coding sequences in pathogen related responses. We previously reported that these two banana *NPR1*-like amino acid coding sequences are only 78% identical (Endah *et al*., 2008). Further, they have only a 42% (*MNPR1A*) and 44% (*MNPR1B*) identity with the third known banana *MdNPR1* (Endah, unpublished data) sequence. *MNPR1* also has a sequence identity of up to 65% with rice *NPR1*-like homologues but not more than 48% identity to the six identified *Arabidopsis NPR1* homologues (Endah, unpublished data). In our study, we found no differential transcription of the *MNPR1* genes using mutant complementation to the biotrophic pathogen *H. arabidopsidis*, the necrotrophic pathogen *B. cinerea* and the hemi-biotrophic pathogen *P. syringae.* This is contrary to the observations of native gene transcription in the natural hosts, bananas where differential responses were observed in response to different elicitors and pathogen (Endah *et al*., 2008). When expressed under the control of a constitutive CaMV 35S promoter, an almost identical defence response to these distinct classes of pathogens was observed suggesting that differences in MNPR1 coding sequences do not have any significant impact on the downstream defence response. This confirms recent

findings with the rice *NH1* and *NH3* genes, which share only a 41% amino acid sequence homology, but confer equal resistance to *X. oryzae* when expressed under the control of a constitutive promoter in *Arabidopsis npr1* mutants (Yuan *et al*., 2007, Bai *et al*., 2010). Yuan *et al*. (2007) reported that *NH1*, *NH2* and *NH3* transcripts increased in a time-dependent manner in transgenic *Arabidopisis npr1-1* mutant plants expressing the genes under control of the 35S promoter and following treatment with SA, methyl jasmonte (MeJA), ethylene (ET) or *Xanthomonas oryzae* (Yuan *et al*., 2007).

Observations from out study therefore suggest that *MNPR1* expression might be sensitive to the promoter and plant host used . The importance of native promoters in expression has been clearly shown with the rice *NH1* and *NH3* gene. When expressed in rice under the control of the CaMV 35S promoter, only *NH1* conferred enhanced resistance to *X. oryzae*. Yuan *et al*. (2007) therefore suggested that the rice *NH1* gene is the true *NPR1* orthologue with a positive role in defence response. However, recently Bai *et al*. (2010) reported that *NH3* also enhances resistance to *X. oryzae* when over-expressed in rice but under the control of its native promoter. Our studies in banana (Endah *et al*., 2008; 2010) together with the complimentation study in *Arabidopsis* confirms the "true" NPR1 nature of *MNPR1A* and *MNPR1B* since both banana *NPR1*-like genes were equally active as positive pathogen defence regulators to a broad range of pathogens in the *Arabidopsis* system. Their differential activity therefore has to be confirmed in transgenic banana over-expressing the two *MNPR1*s under the control of the CaMV 35S as well as under their native promoters. The action of a native promoter might also be required for any differential response in banana towards pathogens and elicitors to fine-tune the overall defence mechanism of banana (Endah *et al*., 2008).

In summary, our study has shown that MNPR1A and MNPR1B induce *PR-1* gene expression and restore pathogen resistance similarly in *Arabidopsis npr1* mutant plants. However,

differences in coding sequence between *MNPR1A* and *MNPR1B* play no significant role in gene activity. Future work will therefore focus on isolation and characterization of native promoter sequences of *MNPR1A* and *MNPR1B* for testing the role of these promoters in differential *MNPR1* response to pathogens. Also existence and expression of the two *MNPR1*s will be investigated in banana cultivars with different genomes to show if the two banana M*NPR1* sequences are specific for the AAA banana genome or are a result of gene duplication and components of a greater banana *NPR1* gene family with differential function.

Materials and methods

MNPR1 cloning and plant transformation

MNPR1 coding sequences were cloned individually as a *BamH*I/*Xba*I fragment between a double CaMV35S promoter and a CaMV terminator sequence in the vector p*LBR19* and coding sequences with the promoter and terminator sequences were finally cloned into the plasmid p*BIN19* (Bevan, 1984) to create an expression vector for *Agrobacterium* transformation. Plasmids containing the *MNPR1* sequences in the right orientation, together with plasmid p*Bin19-LBR* without a *MNPR1* insertion (serving as control plasmid), were used to transform *Agrobacterium* LBA4404 competent cells using the freeze-thaw method as described by Chen *et al*. (1994). Resultant *Agrobacterium* strains harbouring the plasmids were designated: p*LBA-MNPR1A*, p*LBA-MNPR1B*, and p*LBA-LBR* and used to transform *Arabidopsis npr1-2* mutants (donated by Dr. Xinnian Dong, USA) as described by Clough and Bent (1998). Selfed progenies from these transformed plants were screened over four kanamycin selection stages as described by Clough and Bent (1998) to obtain homozygous transgenic *Arabidopsis* lines. The presence and correct orientation of the gene in these lines was also verified via PCR using their individual genomic DNA together with gene specific

primers and through sequencing. Transgenic plants were designated *npr1*:M*NPR1A*, *npr1*:M*NPR1B*, or *npr1*:*35SCaMV*.

Quantitative Real Time-Polymerase Chain Reaction

QRT-PCR was performed to measure the amount of the *PR-1* transcripts in both pathogen-inoculated and non-inoculated plants. Also, the presence of *MNPR1* transcripts for each plant was confirmed via PCR and using complimentary DNA (cDNA) synthesized from RNA isolated from these lines. Total RNA extraction in all samples to be analyzed was performed using the Trizol reagent (Invitrogen Life Technologies, San Diego, California, USA) following the manufacturer's instructions. Contaminating DNA was removed by treating RNA samples with TurboDNA free reagent (Ambion, UK) according to the manufacturer's protocol. First-strand cDNA was synthesized from 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* (AT2G14610). The endogenous control primer was designed from the *Arabidopsis ACTIN2* gene (At3g18780). It was important that resultant primers were specific to only the gene of interest and this was further verified via PCR and using the other genotypes as the negative control during the various PCR reactions.

The Sybrgreen master mix (Roche Diagnostics, UK) was used for the qRT-PCR as follows: 5 µL of cDNA template, 1 µL of 10 µM primers (Table SI), 10 µL SYBR-Green I master mix, and 3 µL nuclease-free water were added into respective wells in a 96 welled-RT-PCR micro-titre plate. 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. Non-template control reactions containing water instead of cDNA as template were included into the reaction. The qRT-PCR reaction was set up at a primer annealing temperature of 60°C for 5 s with an amplification phase of 40 cycles and following the instructions outlined in the Sybrgreen master mix (Roche Diagnostics, UK) protocol.

The Applied Biosystem's software program was applied to determine the relative amount of transcript in each sample. The Applied Biosystems, User Bulletin No. 2, (2000), and steps described in Livak and Schmittgen (2001) were applied for data analysis. The basal relative transcript amount for each sample was expressed relative to the *ACTIN2* gene and after pathogen infection, relative to their basal transcript amounts at time point 0 h (Applied Biosystems, User bulletin No.2, 2001; see detailed description in Endah *et al*., 2008).

Pathogen growth and plant treatment

Unless stated otherwise, five-week old *Arabidopsis* wild-type, *npr1*:*35SCaMV*, *npr1*:*MNPR1A44*, *npr1*:*MNPR1A56*, *npr1*:*MNPR1A95*, *npr1*:*MNPR1B1*, *npr1*:*MNPR1B12* and *npr1*:*MNPR1B15* plants grown under a 10 h day/14 h dark regime and a relative humidity of 65 ± 5% were used for these experiments.

Arabidopsis plants were infected with either a virulent strain of *P. syringae* pv. *tomato* DC3000-LuxCDABE (*Pst*-DC3000-Lux; Fan *et al*., 2008), the avirulent *P. syringae* pv. *tomato* avrRPM1 (*Pst*avrRPM1; JIC, Norwich, UK), the necrotrophic fungus *B. cinerea* B05.10 (kindly provided by Dr Henk-jan Schoonbeek, JIC Norwich, UK), or the biotrophic fungus *H. arabidopsidis* (Sainsbury laboratory, Norwich, UK). Plants of three independent *npr1-2* homozygous transgenic *Arabidopsis* lines expressing either *MNPR1A*

(*npr1*:*MNPR1A44*, *npr1*:*MNPR1A56*, *npr1*:*MNPR1A95*), or *MNPR1B* (*npr1*:*MNPR1B1*, *npr1*:M*NPR1B12*, and *npr1*:*MNPR1B15*) coding sequences as well as transgenic *npr1-2* plants carrying the empty vector alone (*npr1:35SCaMV*), and also wild-type *Arabidopsis* Col-0 plants were finally infected with pathogens.

Infection with P. syringae

A single leaf from each plant was syringe-infiltrated with either 10 mM $MgCl_2$ followed by a secondary infiltration of three additional leaves per plant after 48 h with virulent *Pst*-DC3000-Lux ($5x10^5$ cfu.mL^{-1} in 10mM $MgCl_2$). This represented the non-SAR induced treatment (MV treatment). In the SAR-induced treatment (AV), *Pst*avrRPM1 ($5x10^6$ cfu.mL^{-1} in 10 mM $MgCl_2$) was used for primary infiltration of a single leaf per plant followed by the secondary challenge of three additional leaves with *Pst*-DC3000-Lux. Growth of *Pst*-DC3000-Lux was determined quantitatively 48 h after the secondary challenge using 8mm leaf discs excised from *Pst*-DC3000-Lux-infiltrated leaves as described by Fan *et al*. (2008). A total of 12 plants were used for each time point. The experiment was repeated twice with independent samples. Samples for RNA extraction were harvested 48 h after the primary and secondary infections, frozen in liquid nitrogen and stored at -70^oC.

Infection with B. cinerea

B. cinerea B05.10 (Büttner *et al*., 1994) was cultured at 20^oC on malt extract agar (30 gL^{-1}) and yeast extract (2 gL^{-1}) medium (MEYA; Oxoid, UK) as described by Schoonbeeck *et al*. (2003). For each plant to be infected, six leaves were used, and 5 µL droplets of either a 2.5 x 10^5 spores/mL *B. cinerea* B05.10 inoculum (resuspended in ¼ strength MEYA liquid medium), or a 1/4 strength MEYA liquid medium (controls) was dropped onto the upper surface of each leaf using a pipette. Trays containing plants were covered with a plastic dome to maintain high humidity.

Lesions that developed 72 hpi on leaves were measured using a digital caliper. For each data set, lesions from six plants and a total of six leaves per plant were measured. Plants for qRT-PCR were sprayed with either the inoculums or a control solution, and samples for qRT-PCR were harvested 48 h post treatment and quickly frozen in liquid nitrogen. The experiment was repeated twice with independent samples.

Infection with H. arabidopsidis

Using a pressure pump set at 10 psi, three-week old soil-grown *Arabidopsis* plants were sprayed either with 5 µL of distilled water (control) or a freshly prepared 2.5 x 10^4 spores/mL *H. arabidopsidis* inoculums, cultured and maintained as described by Rairdan *et al*. (2001). Whole *Arabidopsis* rosette leaves were harvested from plants 7 dpi and immediately placed in 50 mL Falcon tubes (six rosettes per tube, a total of two tubes per genotype) and used for trypan blue staining following the method described by Koch and Slusarenko (1990). Individual leaves (four each) were mounted on microscope slides in 80% glycerol (v/v) for conidiophore counting under a light microscope. Samples for qRT-PCR were harvested 48 h post-treatment and quickly frozen in liquid nitrogen. The above experiment was repeated twice.

Statistical analysis

The statistical difference in each experiment over time for each treatment and between genotypes was analysed by One-way ANOVA and the Tukey highest square difference (HSD) test using the Statistical analysis software-SAS® (SAS, USA). The Student's t-test was also used to determine the statistical difference between two independent smaples. The cut-off value was set at $P<0.05$ for samples that were significantly different from each other.

Acknowledgement

Our appreciation goes to Prof Nick Brewin, Dr Jun Fan and Dr Henk-jan Schoonbeeck (John Innes Centre, UK) and Dr Georgina Fabro (Sainsbury Laboratory, UK) for the provision of facilities and for technical advice throughout the course of this work, to the Rockefeller Foundation and the University of Pretoria for funding, and to Prof. Xinnian Dong (Duke University, USA) for donation of mutants, and to Prof David Lawlor for critical review of the manuscript.

Reference

Applied Biosystems. 2001. ABI PRISM 7700 sequence detection system. User Bulletin No. 2.

Bai, W., Chern, M., Ruan, D., Canlas P. E., Sze-to, W. H., Ronald, P. C. 2011. Enhanced disease resistance and hypersensitivity to BTH by introduction of an *NH1*/*OsNPR1* paralog. Plant Biotechnol. J. 9:205-215.

Bergeault K, Bertsch C, Merdinoglu D, Walter B. 2010. Low level of polymorphism in two putative NPR1 homolgs in the Vitaceae family. *Direct* 5:9-21.

Büttner, P., Koch, F., Voigtk K., Quidde, T., Risch, S., Blaich, R., Bruckner, B., and Tudzynski, P. 1994. Variations in ploidy among isolates of *Botrytis cinerea*: Implications for genetic and molecular analyses. Curr. Genet. 25:445-50.

Canet, J. V., Dobón, A., Roig, A., and Tornero, P. 2010. Structure-function analysis of *npr1* alleles in *Arabidopsis* reveals a role for its paralogs in the perception of salicylic acid. Plant Cell Environ. 33:1911-1922.

Cao, H., Bowling, S. A., Gordon, S.A., and Dong, X. 1994. Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell 6:1583-1592.

Cao, H., Glazebrook, J., Clarke, J. D., Volko, S., and Dong, X. 1997. The *Arabidopsis NPR1* gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell 88:57-63.

Chen, H., Nelson, R. S., and Sherwood, J. L. 1994. Enhanced recovery of transformants of *Agrobacterium tumefaciens* after freeze-thaw transformation and drug selection. Biotech. **16**:664–670.

Clough, S. J., and Bent, A. F. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. 16:735-743.

Endah, R., Beyene, G., Kiggundu, A., Van den Berg, N., Schlüter, U., Kunert, K., and Chikwamba, R. 2008. Elicitor and *Fusarium*-induced expression of *NPR1*-like genes in banana. Plant Physiol. Biochem. 46:1007-1014.

Endah, R., Coutinho, T., and Chikwamba, R. 2010. .*Xanthomonas campestris* pv *musacearum* induces sequential expression of two *NPR-1* like genes in banana. Aspects Appl. Biol. 96., Agriculture: Africa's "engine for growth" Plant Science and Biotechnology holds the key., 325-330.

Fan, J., Crooks, C., and Lamb, C. 2008. High throughput quantitative luminescence assay of the growth *in planta* of *Pseudomonas syringae* chromosomally tagged with *Photorhabdus luminescens lux*CDABE. Plant J. 53:393-399.

Glazebrook, J., Rogers, E. E., and Ausubel, F. M. 1996. Isolation of *Arabidopsis* mutants with enhanced disease susceptibility by direct screening. Genetics 143:973-982.

Hwang, S. C., and Ko, W. H. 2004. Cavendish banana cultivars resistant to *Fusarium* wilt acquired through somaclonal variation in Tawain. Plant Dis. 88:580-588.

Koch, E., and Slusarenko, A. J. 1990. *Arabidopsis* is susceptible to infection by a downy mildew fungus. Plant Cell 2:437-455.

Liu G, Holub EB, Alonso JM, Ecker JR, Fobert PR. 2005. An *Arabidopsis NPR1*-like gene, *NPR4*, is required for disease resistance. *Plant J*. 41:304-318.

Livak, K. J., and Schmittgen, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods 25:402-408.

Pieterse, C. M., and van Loon, L. C. 2004. *NPR1*: the spider in the web of induced resistance signaling pathways. Curr. Opin. Plant Biol. 7:456-464.

Rairdan, G. J., Donofrio, N. M., and Delaney, T. P. 2001. Salicylic acid and NIM1/NPR1-independent gene induction by incompartible *Peronospora parasitica* in *Arabidopsis*. Mol. Plant-Microbe Interact. 14:1235-1246.

Sandhu, D., Tasma, M. I., Frasch, R., and Bhattacharyya, M. K. 2009. Systemic acquired resistance in soybean is regulated by two proteins orthologous to *Arabidopsis NPR1*. *Plant Biol*. 9:105-119.

SAS Institute Inc. SAS® v9.2. Cary., NC: SAS Institute Inc.

Schoonbeek, H., van Nistelrooy, J. G. M., and de Waard, A. 2003. Functional analysis of ABC transporter genes from *Botrytis cinerea* identifies BcatrB as a transporter of eugenol. *European J. Plant Pathol.* 109:1003–1011.

Shi, Z., Maximova, S. N., Liu, Y., Verica, J., and Guiltinan, M. J. 2010. Functional analysis of the *Theobroma cacao NPR1* gene in *Arabidopsis*. *Plant Biol*. 10:248-265.

Stover, R. H., and Buddenhagen, I. W. 1986. Banana breeding: polyploidy., disease resistance and productivity. *Fruits* 41:175-191.

The *Arabidopsis* Genome initiative 2000. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* 408:796-815.

Wally, O., Jayaraj, J., and Punja, Z. K. 2009. Broad-spectrum disease resistance to necrotrophic and biotrophic pathogens in transgenic carrots (*Daucus carota* L.) expressing an *Arabidopsis NPR1* gene. *Planta* 231:131-141.

Yuan, Y., Zhong, S., Li, Q., Zhu, Z., Lou, Y., Wang, L., Wang, J., Wang, M., Li, D., Yang, D., and He, Z. 2007. Functional analysis of rice *NPR1*-like genes reveals that *OsNPR1/NH1* is the rice orthologue conferring disease resistance with enhanced herbivore susceptibility. *Plant Biotech. J*. 5:313-324.

Zhao, J-T., Huang, X., and Chen, Y-P. 2009. Molecular cloning and characterization of an ortholog of *NPR1* gene from Dongguan Dajiao (*Musa* spp. ABB). *Plant Mol. Biol. Rep*. 27:243-249.

Supplementary material

Table S1 Primer sequences used for amplification of *AtPR-1* and *MNPR1* genes

Primer name	Primer sequence
qM*NPR1*A_FF	GCTCGACAGATTTTGCTTATC
qM*NPR1*A_RR	GGAAGACAAAGCAGACTTGTTG
qM*NPR1*B_FF	CCTCCTTGGTATTCTGGACA
qM*NPR1*B_RR	CTTCTTCTCTAGGGTGATGG
qPR1_FF	CGGAGCTACGCAGAACAACT
qPR1_RR	CTCGCTAACCCACATGTTCA
qACTIN2_FF	AGTGGTCGTACAACCGGTATTGT
qACTIN2_RR	GATGGCATGGAGGAAGAGAGAAAC

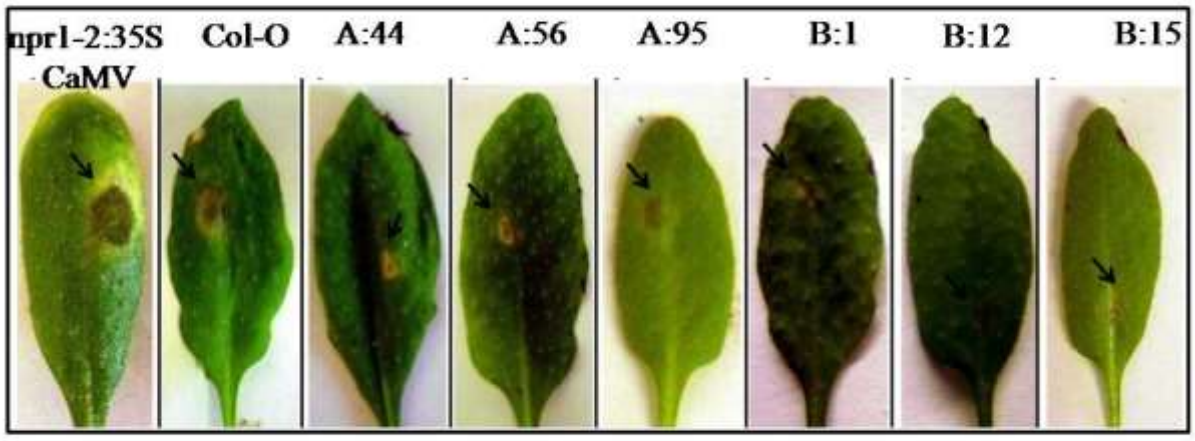

Figure S1 Pictures of typical Botrytis lesions on *Arabidopsis* leaves 72 h post *Botrytis* infection. Leaves (six per plant) of eight different *Arabidopsis* genotypes were infected with a 2.5 x 10^5 spores/mL of an inoculum of *B. cinerea* B05.10. Pictures were taken 72 hpi to illustrate the lesions formed Arrows on the leaves indicate the

point of lesion initiation. The experiment was repeated twice using independent sets of plant material treated in the same manner described above.

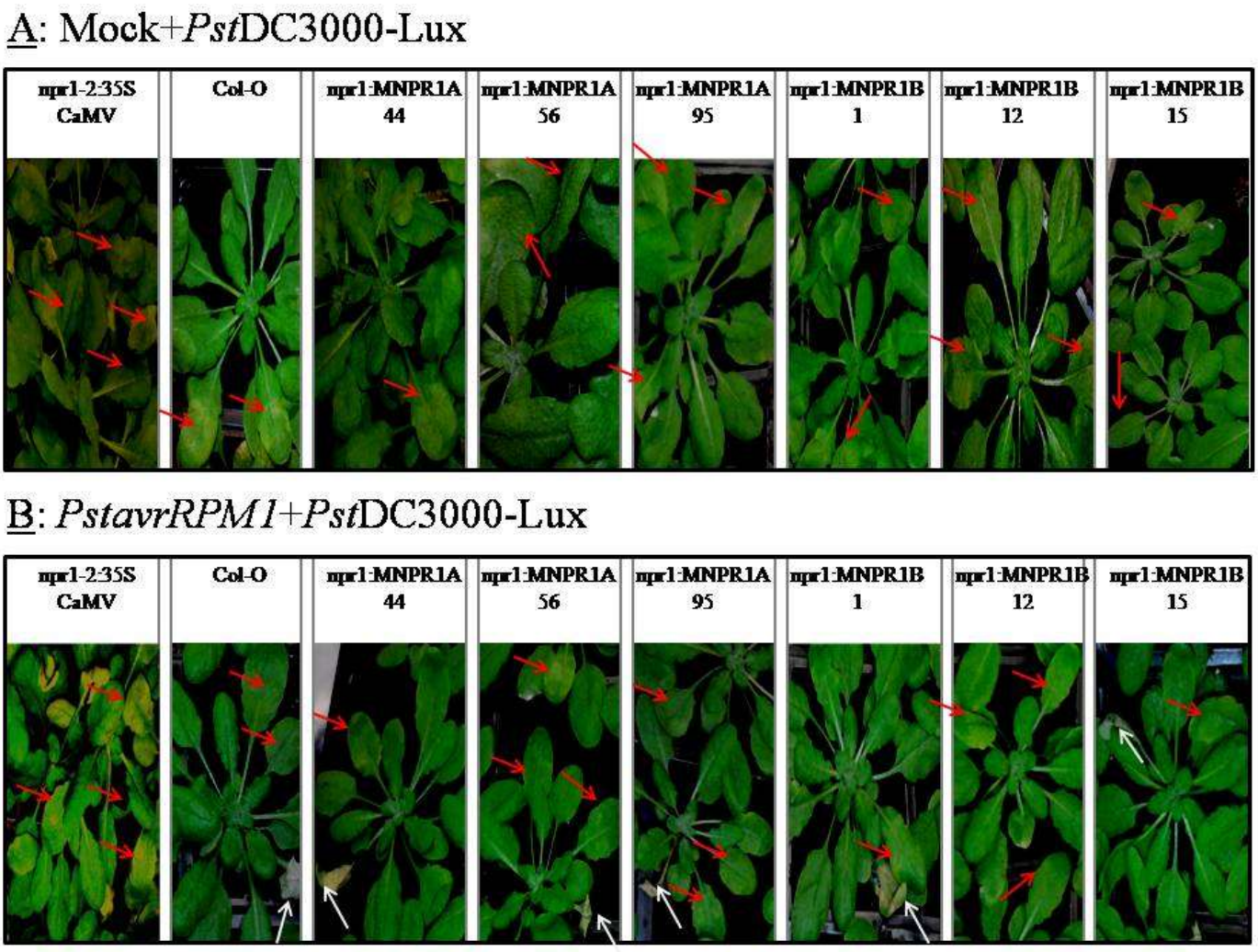

Figure S2 Pictures of infected plants 48 h post *Pseudomonas syringae* infection. A single leaf of eight different *Arabidopsis* genotypes were infiltrated primarily with either a 10 mM $MgCL_2$ solution or *Pst*avrRPM1 ($5x10^6$ $cfu.ml^{-1}$ in 10mM $MgCl_2$) inoculum. This was followed 48 later by a secondary infiltration with a *Pst*-DC3000-Lux ($5x10^5$ $cfu.ml^{-1}$ in 10mM $MgCl_2$) inoculum of three additional leaves per plant. Pictures of infected plants were taken digitally 48 h after the secondary infiltration. Some of the *Pst*DC3000-Lux infected leaves (red arrows) and *Pst*avrRPM1-infected leaves (white arrows) are represented (B and C). The experiment was repeated twice using independent sets of plant material treated in the same manner described above.