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# Molecular Cloning and Characterization of an Ortholog of *NPR1* Gene from Dongguan Dajiao (*Musa* spp. ABB)

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**Abstract** The nonexpresser of pathogenesis-related gene 1 (*NPR1*) plays a pivotal role in systemic acquired resistance in plants. In this study, a novel full-length *NPR1*-like gene, designated *MdNPR1* (accession number FJ357442), was isolated by RACE-PCR from cv. Dongguan Dajiao (*Musa* spp. ABB), a local banana cultivar known to be resistant to *Fusarium oxysporum* fsp. *cubense* (FOC) race 4. Sequence alignment showed that MdNPR1 contained an ankyrin repeat domain and a broad complex, tramtrack, and bric-a-brac (BTB) domain. Semiquantitative reverse transcription polymerase chain reaction revealed that *MdNPR1* could be constitutively expressed at low levels in both of the FOC race 4-susceptible cultivar Fenjiao (*Musa* spp. ABB) and the resistant cultivar Dongguan Dajiao. However, *MdNPR1* could be induced by exogenous application of salicylic acid in cv. Dongguan Dajiao, but not in cv. Fenjiao. Moreover, the accumulated level of *MdNPR1* transcripts in cv. Dongguan Dajiao was higher than that in cv. Fenjiao when plants were treated with FOC race 4 inoculation. Our results implied that *MdNPR1* might represent as a promising candidate for engineering resistant to broad-spectrum pathogen in banana.

**Keywords** Dongguan Dajiao (*Musa* spp. ABB) · *Fusarium oxysporum* fsp. *Cubense* · NPR1 · Resistant · Salicylic acid

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**Abbreviations**

| | |
|---|---|
| BTB/POZ | broad complex, tramtrack, and bric-a-brac/pox virus and zinc finger |
| FOC | *Fusarium oxysporum* fsp. *Cubense* |
| NPR | nonexpresser of pathogenesis-related gene |
| PR | pathogenesis-related |
| ORF | open reading frame |
| RACE | rapid amplification of cDNA ends |
| SA | salicylic acid |
| SAR | systemic acquired resistance |
| TGA | subclass of bZIP transcription factors that bind to cognate *as-1*-type (TGACGT) *cis*-promoter elements |

## Introduction

Systemic acquired resistance (SAR) is long-lasting and appears to be effective against a broad spectrum of pathogens (Durrant and Dong 2004). Extensive studies have shown that the nonexpresser of pathogenesis-related gene 1 (*NPR1*) functions as the key regulator of salicylic acid (SA)-mediated SAR in *Arabidopsis*. The NPR1 protein contains a bipartite nuclear localization sequence and two protein–protein interaction domains: an ankyrin repeat domain and a broad complex, tramtrack, and bric-a-brac/pox virus and zinc finger (BTB/POZ) domain (Cao et al. 1997). Nuclear localization of NPR1 is essential for its function in inducing pathogenesis-related (*PR*) gene expression (Kinkema et al. 2000). In response to SA, NPR1 moves to the nucleus where it interacts with TGA transcription factors to induce defense gene expression, thus activating SAR (Durrant and Dong 2004). NPR1 also mediates cross-talk between the SA signaling pathway and the jasmonic acid (JA) signaling pathway, and the antagonistic effect of SA on JA signaling requires NPR1 (Spoel et al. 2003). A disease resistance pathway similar to that of the *Arabidopsis NPR1* (*AtNPR1*)-mediated signaling pathway was demonstrated in rice (Chern et al. 2001). Overexpression of *AtNPR1* in *Arabidopsis*, rice, tomato, wheat, and apple have been showed to enhance fungal and bacterial resistance (Cao et al. 1998; Chern et al. 2001; Lin et al. 2004; Makandar et al. 2006; Malnoy et al. 2007). Thus, *NPR1* represents an ideal target for engineering broad-spectrum pathogen resistance in agriculture and encourage the search for functional *NPR1*-like genes in other plant species. Moreover, the study of rice *NPR1* (*OsNPR1*) has revealed that although rice and *Arabidopsis* share conserved defense pathways, the regulation of these pathways and the links to other plant pathways may be quite divergent (Chern et al. 2005). There is a need for the identification, isolation, and characterization of *NPR1* homologues from more species of plants, particularly monocotyledonous plants.

Banana (*Musa* spp.) is among the world's major food crops in tropical and subtropical countries and an important export commodity for producing countries. Banana fruit production is severely limited by several diseases and pests, especially the fungi *Fusarium oxysporum* fsp. *cubense* (FOC), the emergence of race 4 and its dissemination poses an immediate threat. Almost all banana cultivars are highly susceptible to FOC race 4, including cv. Fenjiao (*Musa* spp. ABB), which is a local cultivar in South China. However, the development of pathogen-resistant banana varieties is limited by triploidy and sterility in most commercial cultivars. Genetic transformation has the potential to overcome these constraints by transferring agronomically important single genes. To date, there are few reports on the isolation of banana resistance gene (*R* gene) (Pei et al. 2006; Santy et al. 2007), and no full-length *R* gene has been isolated.

Dongguan Dajiao (*Musa* spp. ABB), a very popular cultivar growing in South China, is highly resistant to FOC race 4 according to field evaluation of banana germplasm to *Fusarium* wilt results (Huang et al. 2005).

In the present study, we isolated a novel full-length *NPR1*-like gene, designated *MdNPR1*, from cv. Dongguan Dajiao and analyzed the deduced peptide sequence with regard to the domains required for function in *Arabidopsis*. We also preliminarily investigated the expression of *MdNPR1* in leaves of cvs. Dongguan Dajiao and Fenjiao during FOC race 4 inoculation and treatment of SA.

## Materials and Methods

### Plant and Fungal Material

Tissue culture-derived plantlets of cvs. Dongguan Dajiao (*Musa* spp. ABB) and Fenjiao (*Musa* spp. ABB) were kindly provided by the China Banana Germplasm Nursery in the Fruit Research Institute, Guangdong Academy of Agricultural Sciences. FOC race 4 was provided by the Guangzhou Agricultural Scientific Research Institute.

### Genomic DNA Extraction and Degenerate PCR

Genomic DNA was isolated from the young leaves of a greenhouse-grown ex vitro plantlet of cv. Dongguan Dajiao using a modified cetyl trimethylammonium bromide (CTAB) method (Stewart and Via 1993). A pair of degenerate primers was designed based on the region conserved in the *Arabidopsis*, tobacco, tomato, rice, and maize *NPR1* gene nucleotide sequence: F1 (5′-AGGCAYTGGAYTCDGAT GATGTTGA-3′) and R1 (5′-TCTYTHCKCATYGCAGCC AKRTGRAG-3′). Touch-Down PCR was used for amplification: Genomic DNA was denatured at 94°C for 5 min, followed by two cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C, followed by two cycles of the above conditions with the annealing temperature decreasing by 2°C every two cycles, followed by 25 cycles of 30 s at 94°C, 30 s at 52°C, and 1 min at 72°C with final extension at 72°C for 5 min by using a DNA thermal cycler (Biometra Thermocycler, Germany). The purified product was cloned into pMD-20 T vector (TaKaRa) and sequenced by Sangon Company (Shanghai, China) using an ABI 3770 DNA sequencer.

### Isolation of the Full-Length *MdNPR1* cDNA Using RACE-PCR

Total RNA was isolated from young leaves of cv. Dongguan Dajiao using the modified CTAB/NaCl method (Asif et al. 2000). Total RNA was treated with the DNaseI (RNase Free) (TaKaRa) to avoid the genomic DNA contamination. The 3′-Full RACE Core Set Kit (TaKaRa) was used for rapid amplification of 3′ cDNA ends following the manufacturer's instructions. The gene-specific primer 3′ GSP (5′-CGCTGAGTTGTTAGACCTTGGGTCAGC-3′), selected from the Touch-Down PCR product, was used as the sense primer, and the 3′ sites Adaptor Primer provided with the kit was used as the antisense primers. The 5′-RACE was performed using BD SMART™ RACE cDNA Amplication Kit (BD Biosciences) according to manufacturer's instructions. The Touch-Down PCR and 3′-RACE-PCR products were used as template to design gene-specific primers. Briefly, the D5R (5′-GGGGGTGCCCTTCTGG AGGTAGAACACATC-3′) and UPM (10× Universal Primer A Mix provided with the kit) were used for first round PCR. GSP1 (5′-CATTGCAGCCAGATGAAGTGGTGTG-3′) and NUP (Nested Universal Primer A provided with the kit) were then used for nested PCR. According to the sequence obtained by the first 5′-RACE-PCR, we designed another nested gene-specific primer GSP2 (5′-ATCATCCGAATCC

AGGGCTATGTGG-3′) for the second 5′-RACE-PCR. Then the third 5′-RACE-PCR was done with the third nested gene-specific primer GSP3 (5′-TGAGACAAGCTCCGCGATCT GAAAG-3′). Amplification conditions were the same as above with the D5R as the primary gene-specific primer and GSP2 and GSP3 as the nested gene-specific primers.

According to the 3′-RACE and 5′-RACE results, the primers F2 (5′-TTCTACGCCATGCCTAATCC-3′) and R2 (5′-CTCTTGCTGCATCGTTTTTG-3′) were used to obtain the full-length cDNA. The full-length cDNA was amplified by reverse transcription polymerase chain reaction (RT-PCR) using the total RNA as the temple isolated from cvs. Dongguan Dajiao and Fenjiao.

### Isolation of *MdNPR1* Gene from Dongguan Dajiao Genomic DNA

The specific primers F2 and R2 were used for amplification of cv. Dongguan Dajiao *MdNPR1* gene from genomic DNA. The PCR mixture contained 20 pmol of each primer, 100 ng genomic DNA, 2 mM of each dNTP, and 2.5 U of LA Taq DNA polymerase (TaKaRa) in a 50 μL reaction volume. PCR amplification was carried out with 30 cycles of 10 s at 98°C, 4 min at 68°C. The purified product was cloned into pMD-20T vector (TaKaRa) and sequenced.

### Sequence Alignment and Phylogenetic Analysis

RACE 5′ and 3′ overlapping sequences were assembled by DNAMAN version 5.29 software (Lynnon Biosoft Company, USA) to obtain full-length sequence. ORF was analyzed using ORF Finder at NCBI (http://www.ncbi.nlm.nih.gov/gorf/gor.html). The deduced amino acid sequence was blasted the CDD database to find the conserved domains. The molecular weight and isoelectric point of the encoded protein was predicted by the Expasy site (http://www.expasy.ch/tools/pi_tool.html). The BLAST search program (http://www.ncbi.nlm.nih.gov/BLAST) was used to search for protein sequences homologous to NPR1. Multiple sequence alignment was done by CLUSTALX (version 1.83), CLUSTALX-produced alignment file was formatted using BOXSHADE program (http://www.ch.embnet.org/software/BOX_form.html) (Liu et al. 2002). Phylogenetic analysis and construction of a neighbor-joining tree were performed by using the MEGA 4.0 software by using the bootstrap method with 1,000 bootstrap iterations (Felsenstein 1985).

### Treatment with Salicylic Acid and *F. oxysporum* fsp. *cubense* Race 4

Tissue culture-derived plantlets of cvs. Dongguan Dajiao and Fenjiao were acclimatized and transferred to the greenhouse and allowed to grow for about 2 months, until they attained the desired size. Plants, about 20 cm in length with six to eight leaves, were selected and divided into two groups and subjected to either SA treatment or FOC inoculation. SA treatment was performed by spraying leaves at concentrations of 1.0 mM in distilled $H_2O$. For FOC inoculation, we used the double-compartment or “double-tray” technique as described by Mak et al. (2004) to safeguard against the pathogen, its containment, and preventing its distribution and release during inoculation. The technique requires a double-tray set up—a perforated inner tray containing sterilized river sand to grow hardened tissue-cultured plantlets and a larger outer containment tray for collecting surplus Hoagland nutrient solution and pathogen wash-outs. This double-compartment or “double-tray” technique was adequate for differentiating between tolerant and susceptible plant lines. All treated/inoculated leaf samples were collected at 0, 4, 8, 12, 24, 36, 48, 60, and 72 h and immediately frozen in liquid nitrogen, followed by storage in a −80°C freezer until RNA extraction.

### Semiquantitative RT-PCR

Total RNA was extracted from leaves after SA or FOC treatment. First strand cDNA was synthesized using the Reverse Transcriptase M-MLV (RNase H−) (TaKaRa) as per manufacturer’s protocol. The primers F3 (5′-GATAA GGCTATGGTGGAAGA-3′) and R3 (5′-CGCCCTAGTT AGTCTCCTACAT-3′) were designed to analyze the expression pattern. The PCR conditions were 94°C for 1 min, followed by 30 cycles of amplification (94°C for 30 s, 55°C for 30 s, 72°C for 1 min), then 72°C for 5 min. The amplified fragment of *MdNPR1* was 555 bp in length. The actin1 (accession number AF285176) was amplified using the primers ActinF (5′-AAGGATATGCCCTCCCTC-3′) and ActinR (5′-CAGAGATGGCTGGAAGAG-3′) as an internal control. The amplified fragment of banana actin was 299 bp in length. Experiments were repeated at least twice with similar results. Results are from one of these experiments.

## Results

### Isolation of the Full-Length *MdNPR1* cDNA

A 210-bp fragment was amplified using the Touch-Down PCR, and the fragment has an uninterrupted open reading frame (ORF). We found that both the nucleotide and amino acid sequence shared high similarity to the corresponding region of the *NPR1* gene of *Arabidopsis*. The complete cDNA sequence, nominated as *MdNPR1*, was obtained by

3′ and 5′ RACE. The sequence had been submitted to the GenBank (accession number FJ357442).

A full-length *MdNPR1* cDNA contained an ORF of 1,788 bp with a 5′-untranslated region of 127 bp and a 3′-untranslated region of 194 bp terminated by a string of A residues. The *MdNPR1* gene encoded a putative protein of 595 amino acids with a predicted molecular weight of 65.88 kDa and an isoelectric point of 6.28.

## Analysis of the Genomic Structure of *MdNPR1*

The genomic DNA sequence of *MdNPR1* was 4,422 bp in length from start codon to the terminator codon and had been submitted to the GenBank (accession number FJ357443). Comparison of the cDNA sequence and the genomic sequence revealed that there were four exons and three introns in the *MdNPR1* gene, the same as *AtNPR1* and *OsNPR1* (Fig. 1). The size of *MdNPR1* genomic DNA sequence was similar to that of rice (Yuan et al. 2007), but much bigger than *Arabidopsis* (Ryals et al. 1997). Moreover, the position of the introns was identical to that of the *Arabidopsis* and rice gene (Fig. 1), indicating the *NPR1* may conserved both in structure and function.

## Analysis of the Deduced Amino Acid Sequence of MdNPR1

The deduced amino acid sequence of the MdNPR1 had 61% sequence identity (Expect=1*e*−90) with AtNPR1 (Cao et al. 1997) and 63% sequence identity (Expect=2*e*−105) with OsNPR1 (Chern et al. 2005). As in AtNPR1, MdNPR1 contained four ankyrin repeats (amino acids 275–403) and a predicted BTB domain (amino acids 74–175) (Fig. 2a) with 68% sequence identity (Expect=6*e*−22) and 57% sequence identity (Expect=4*e*−11) to the corresponding region of AtNPR1, respectively, which were slightly more conserved than the entire protein. Amino acid crucial for the NPR1 function as defined by genetic mutants, such as npr1-1 (H), npr1-2 (C) (Cao et al. 1997), and nim1-4 (R) (Ryals et al. 1997) were conserved in MdNPR1 (Fig. 2b). In addition, four out of five amino acids required for nuclear localization of AtNPR1 (Kinkema et al. 2000) were conserved in MdNPR1 (Fig. 2b).

## Phylogenetic Analysis

We also retrieved 18 different *NPR1* genes from different plant species through BLASTP searches, including recently reported *NPR1*-like genes from banana (*Musa acuminata* AAA Group) (Endah et al. 2008). Plant *NPR1* genes can be grouped into two clusters (Fig. 3): *MNPR1A* and *MNPR1B* had a closer relationship to rice and barley *NPR1* within the same cluster; however, *MdNPR1* was more closely related to maize *NPR1* in the second cluster, thus suggesting that *MdNPR1* may have a different function than those of *MNPR1A* and *MNPR1B*.

## Effect of Salicylic Acid on Expression of *MdNPR1*

Semiquantitative RT-PCR showed that *MdNPR1* had low levels of expression constitutively in both cvs. Dongguan Dajiao (Fig. 4a, lane 1) and Fenjiao (Fig. 4b, lane 1). However, stronger *MdNPR1* expression in cv. Dongguan Dajiao could be induced by exogenous application of SA, and the induced expression reached a maximum at 8 h (Fig. 4a, lane 3), which was increased approximately twofold to threefold as that at initial time of SA treatment and reduced beyond 12 h (Fig. 4a, lane 4) to the constitutive level of expression (Fig. 4a, lanes 5–9). The pattern of induction expression was similar to that reported in *Arabidopsis*, which is increased approximately twofold by SA treatment (Cao et al. 1998). But *MdNPR1* expression in cv. Fenjiao was not as distinctly elevated as in cv. Dongguan Dajiao by exogenous application of SA (Fig. 4b).

## Expression of *MdNPR1* Induced by FOC Inoculation

As Fig. 4c indicated, FOC inoculation strongly and immediately led to elevated *MdNPR1* transcript levels in cv. Dongguan Dajiao. The induced expression reached a maximum of twofold to threefold with 4–8 h (Fig. 4c, lanes 2 and 3) and then reduced. But the *MdNPR1* in cv. Fenjiao was slightly elevated post 4 h of FOC inoculation (Fig. 4d, lane 2), then reduced to the constitutive levels (Fig. 4d, lanes 3–8). Moreover, induction expression of *MdNPR1* in cv. Dongguan Dajiao by FOC infection was much stronger and faster than that by SA treatment and lasted a longer time.

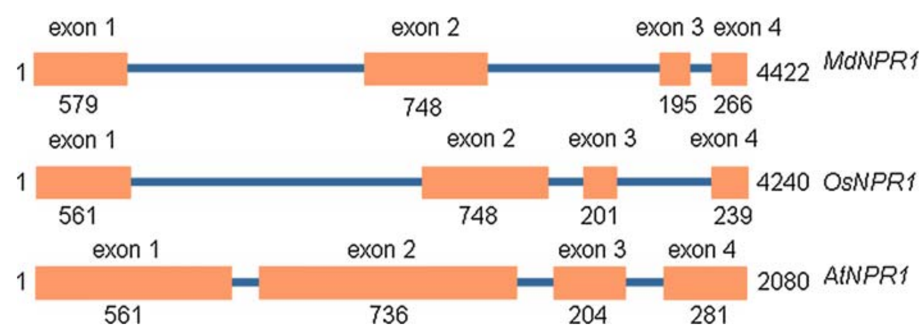


**Fig. 1** The comparison of the genomic structure of *MdNPR1* (accession number FJ357443), *AtNPR1* (accession number U87794), and *OsNPR1* (accession number DQ450948). The length of the exons of *MdNPR1*, *AtNPR1*, and *OsNPR1* genes are indicated below them, respectively

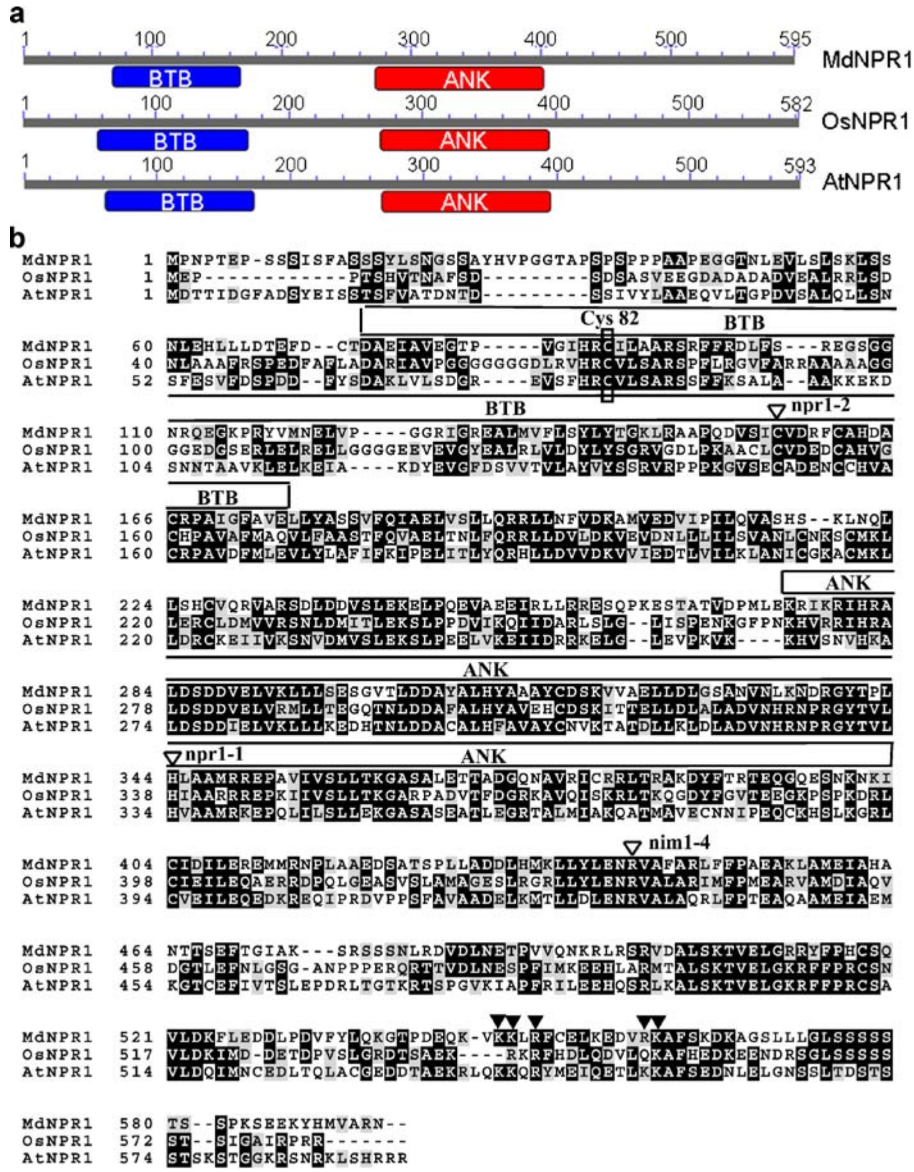


**Fig. 2** Alignment of *MdNPR1* (accession number FJ357442), *AtNPR1* (accession number U76707.1), and *OsNPR1* (accession number AY923983) amino acids sequence. **a** Domain structure of the MdNPR1, OsNPR1, and AtNPR1 protein. An ankyrin repeat domain (ANK) and a BTB domain are indicated. **b** CLUSTALW-produced alignment file was formatted using BOXSHADE program (http://www.ch.embnet.org/software/BOX_form.html). Identical amino acids are highlighted in *black*, while conservative substitutions are marked with *gray*. The protein domains are indicated *above* the sequences. The amino acids changed in npr1-1 (H), npr1-2 (C), and nim1-4 (R) mutants are marked by *open triangles*. Cys82 was indicated by *rectangles*. Amino acids required for nuclear localization of NPR1 in *Arabidopsis* are marked with *filled triangles*

## Discussion

The key regulator of SA-mediated resistance, *NPR1*, is functionally conserved in diverse plant species (Durrant and Dong 2004). Homologs of the *AtNPR1* gene have now been isolated from several plant species (Liu et al. 2002; Zhu et al. 2003; Chern et al. 2005; Meur et al. 2006; Malnoy et al. 2007; Endah et al. 2008), but functional analysis has been carried out primarily only in the model plant *Arabidopsis* and rice.

In this study, we reported a novel full-length *NPR1*-like gene, designated *MdNPR1*, from cv. Dongguan Dajiao. The deduced amino acid sequence of *MdNPR1* had high sequence identity with those of *AtNPR1* and *OsNPR1* and contained both an ankyrin repeat domain and a BTB domain. Sequence comparisons indicated that residues near Cys82, which had been shown to be required for keeping AtNPR1 in the cytoplasm (Mou et al. 2003), were conserved in MdNPR1 (Fig. 2b). Conservation of these structural domains in MdNPR1 indicated they might play a similar function and role in both banana and *Arabidopsis*.

Endah et al. (2008) recently reported that two *NPR1*-like genes, *MNPR1A* and *MNPR1B*, were isolated from banana (*Musa acuminata* AAA group). The deduced amino acid sequence of *MdNPR1* had 65% sequence identity (Expect=1$e$−112) with *MNPR1A* and 65% sequence identity (Expect=9$e$−116) with *MNPR1B*. Based on the phylogenetic tree of all the known NPR1-like proteins from different plants, MNPR1A, MNPR1B, and MdNPR1 were grouped into different clusters, thus suggesting that they may have different functions.

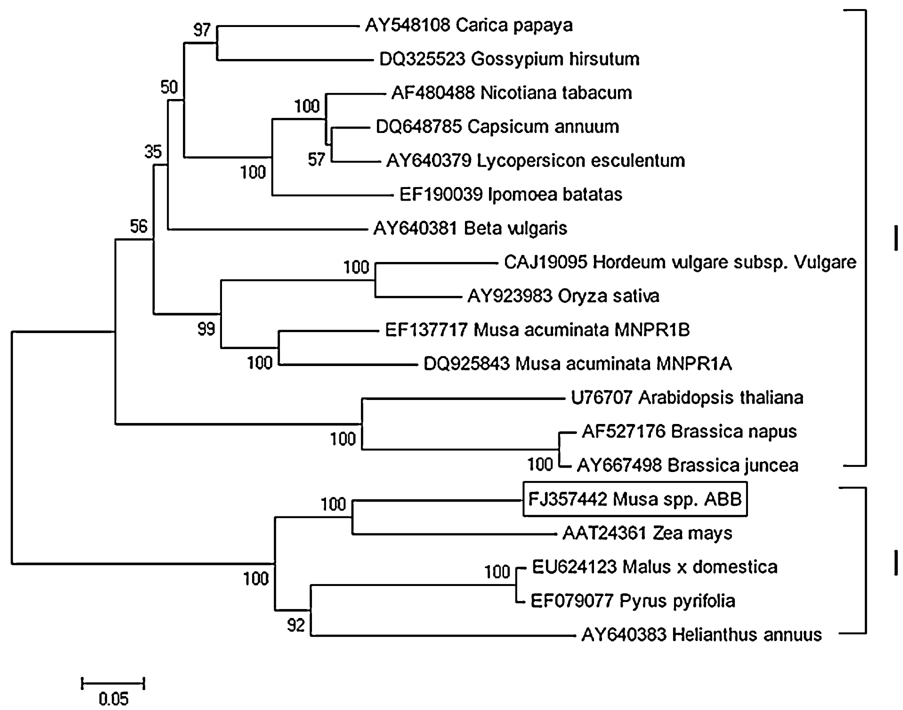


**Fig. 3** Phylogenetic tree of the MdNPR1 and the known NPR1-like protein from other species. GenBank accession numbers are given for each sequence followed by species name. MdNPR1 is indicted by a *rectangle*. The *numbers* on the branches indicate bootstrap values

Endah et al. (2008) have reported that *MNPR1A* was highly expressed following FOC treatment, but not following SA treatment; while *MNPR1B* was highly responsive to SA treatment, but not to FOC treatment. Our results indicated *MdNPR1* was constitutively expressed at low levels in banana, and its level could be further elevated by twofold to threefold after SA treatment and following FOC inoculation in cv. Dongguan Dajiao, which was similar to findings reported in *Arabidopsis* (Cao et al. 1997; Ryals et al. 1997).

However, it was worth noting that SA treatment did not distinctly elevate the *MdNPR1* mRNA levels in cv. Fenjiao, as that in cv. Dongguan Dajiao. Moreover, the *MdNPR1* in cv. Fenjiao was slightly elevated following FOC inoculation, indicating that the *MdNPR1* could be induced in susceptible cultivar. The accumulated level of *MdNPR1* transcripts in cv. Dongguan Dajiao was higher than that in cv. Fenjiao when plants were treated with FOC race 4 inoculation. Similar results also have reported in rice, *OsNPR1* was more rapidly induced in the incompatible (resistant) interactions than in the compatible (susceptible) interactions (Yuan et al. 2007). Our results indicated that different expression of *MdNPR1* in cvs. Dongguan Dajiao and Fenjiao induced by FOC inoculation and SA treatment might be associated to mechanism of the resistant to FOC in cv. Dongguan Dajiao. This mechanism was also confirmed by the fact that the expression of *MdNPR1* in cv. Dongguan Dajiao was faster and stronger during FOC inoculation than during SA treatment.

To confirm this hypothesis, we need to do more studies on structure comparison of the *NPR1* homolog gene from other banana cultivars and their expression pattern following FOC inoculation.

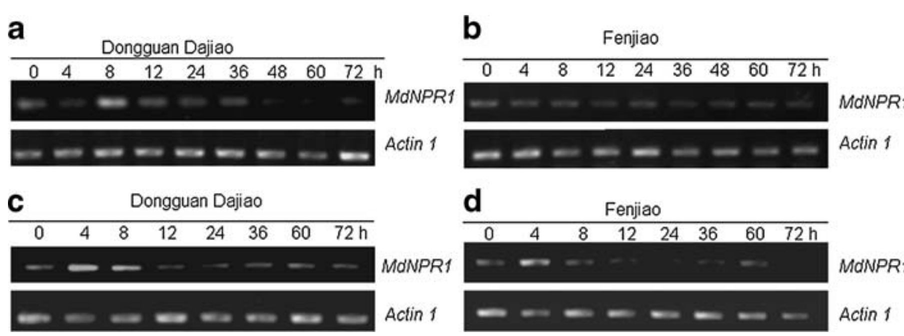


**Fig. 4** Effects of SA treatment and FOC infection on *MdNPR1* expression in Dongguan Dajiao and Fenjiao. Semiquantitative RT-PCR analysis of transcript levels for *MdNPR* in response to 1.0 mM SA treatment in leaves of Dongguan Dajiao (**a**) and Fenjiao (**b**). Induction of *MdNPR1* in leaves of Dongguan Dajiao (**c**) and Fenjiao (**d**) by infection of FOC race 4. Actin1 as an internal control

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