# Over-expression of the citrus gene *CtNH1* confers resistance to bacterial canker disease

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## ARTICLE INFO

## ABSTRACT

Citrus canker is a devastating disease, caused by *Xanthomonas axonopodis* pv. *citri* (*Xac*). It is well established that the *NPR1* gene plays a pivotal role in systemic acquired resistance (SAR) in Arabidopsis. In this study, we report the isolation and characterization of an *NPR1* homolog from citrus, namely *Citrus NPR1 homolog 1* (*CtNH1*). Sequence alignment and phylogenetic analysis indicate that *CtNH1* is closely-related to the Arabidopsis *NPR1* gene and its orthologs from rice, grapevine, and cacao. When over-expressed in citrus, *CtNH1* confers resistance to *Xac* and leads to constitutive expression of the pathogenesis-related (*PR*) gene *chitinase 1* (*Chi1*), suggesting that *CtNH1* is orthologous to *NPR1*.

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## 1. Introduction

Citrus bacterial canker disease is caused by *Xanthomonas axonopodis* pv. *citri* (*Xac*). The bacterium infects plants through stomata and wounds, causing necrotic lesions on the fruits, leaves, and stems, which ultimately affects fruit quality and yield [1,2]. Pathogenicity of *Xac* requires the function of a bacterial type III secretion system that delivers the so-called type III effectors into the host cell [2]. Since its first discovery in Florida in 1912, citrus canker has been a major threat to the $9 billion Florida citrus industry. Most citrus varieties are either highly or moderately susceptible to the disease [2]. Despite the implementation of long term eradication programs removing millions of high value trees, *Xac* was spread to most citrus producing areas in Florida [3]. The eradication program was suspended in 2006. Currently, methods used to control the disease have included copper sprays and various cultural practices. However, none of these approaches can efficiently and effectively prevent the spread or outbreak of citrus canker.

After successful infection by pathogens, some plant species such as Arabidopsis, tobacco, and cucumber can initiate a secondary immunity in systemic tissues known as systemic acquired resistance (SAR) [4]. SAR is characterized by the accumulation of salicylic acid (SA), induction of a large number of pathogenesis-related (*PR*) genes, and broad, long-lasting disease resistance. In addition to pathogen infection, SAR can also be induced by SA and its analogs such as 2,6-dichloroisonicotinic acid (INA), and benzo (1,2,3)-thiadiazole-7-carbothioic acid S-methylester (BTH). The Arabidopsis gene, *NPR1* (also known as *NIM1* and *SAI1*), is a key regulator of SAR [5—10]. Mutations in this gene result in plants that are no longer responsive to SAR induction. The *PR* gene expression and disease resistance are both abolished in *npr1* mutants. *NPR1* encodes a protein with a BTB (bric à brac, tramtrack, broad-complex) domain, four ankyrin repeats, and a bipartite nuclear localization signal (NLS) [9,11]. In untreated tissues, NPR1 forms an oligomer through intermolecular disulfide bridges that involve the conserved cysteine residues Cys82 and Cys216 [12]. Upon stimulation with SAR signals, the NPR1 complex dissociates and the released monomeric NPR1 accumulates in the nucleus leading to the activation of defense genes.

In the sequenced Arabidopsis genome, five additional genes are predicted to encode proteins similar to NPR1. These genes are named *AtNPR2* to *AtNPR6* (*NPR1* is also called *AtNPR1*) [13—17]. It has recently been demonstrated that AtNPR3 and AtNPR4 function as SA receptors as well as adapters that link NPR1 with the CUL3 E3 ligase for degradation [18]. *AtNPR5* and *AtNPR6*, also known as *AtBOP1* and *AtBOP2*, play roles in the regulation of proper leaf and flower morphogenesis [15—17]. Among these paralogs, *AtNPR2* is the most phylogenetically-related to *AtNPR1*. However, the precise

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role of *AtNPR2* remains to be elucidated. The presence of multiple, functionally diverse homologs complicates the identification of NPR1 orthologs in other plant species.

Owing to the broad, long-lasting disease resistance nature of SAR, *AtNPR1* has been used as an important source for engineering resistance in agronomically important crops. Over-expression of *AtNPR1* in Arabidopsis enhances disease resistance to bacterial and fungal pathogens [19,20]. When over-expressed in rice, *AtNPR1* provides resistance to bacterial blight disease caused by *Xanthomonas oryzae* pv. *oryzae* [21,22]. Moreover, transgenic tomato plants with elevated *AtNPR1* expression display an enhanced resistance to bacterial wilt and Fusarium wilt, and a moderate degree of resistance to gray leaf spot and bacterial spot [23]. In wheat, over-expression of *AtNPR1* results in resistance to Fusarium head blight disease caused by the fungal pathogen *Fusarium graminearum* Schwabe [24]. *AtNPR1* has also been shown to provide resistance to citrus canker [25].

*AtNPR1* orthologs and homologs have also been isolated for disease control from diverse plant species including rice, grapevine, cacao, apple, banana, and cotton [26–32]. Similar to *AtNPR1*, over-expression of the rice ortholog, *OsNPR1/NH1*, confers resistance to bacterial blight disease, whereas transgenic plants over-expressing an apple homolog of *NPR1*, *MpNPR1-1*, exhibit a resistance to both bacterial and fungal pathogens [26–28]. More recently, over-expression of the grapevine ortholog of *AtNPR1*, *VvNPR1*, enhances resistance to powdery mildew [32]. Despite this progress, a functional *NPR1* has yet to be identified in citrus.

Here, we report the isolation of an *NPR1* homolog, *Citrus NPR1 homolog 1* (*CtNH1*), from pummelo (*Citrus maxima*). *CtNH1* is phylogenetically-related to *AtNPR1* and its known orthologs. Over-expression of *CtNH1* in citrus confers resistance to *Xac*. Additionally, a combination of phylogenetic analyses and multiple sequence alignment refines the ability to predict *NPR1* orthologs in other plant species.

## 2. Materials and methods

### 2.1. Isolation of a full-length CtNH1 cDNA

Degenerate PCR primers, listed in Suppl. Table 1, were designed according to the conserved region of the NPR1 homologs from Arabidopsis, tobacco, tomato, maize, and rice. After reverse transcription polymerase chain reaction (RT-PCR) amplification using total RNA isolated from the leaf tissues of pummelo, we obtained a cDNA fragment of 440 bp. Gene-specific primers (GSP)-F2, GSP-F3, GSP-R2, and GSP-R3 primers were then designed based on the sequence of this fragment. The 5′-(∼1.5 kb) and the 3′-(∼1 kb) regions of *CtNH1* were obtained by performing 5′ and 3′ RACE using GeneRacer™ kit (Invitrogen, USA) according to the manufacturer's instructions along with the GeneRacer 5′-primer and the GSP-R3, the GeneRacer 3′-primer and the GSP-F3 listed in Suppl. Table 1, respectively. The obtained 5′- and 3′-regions were then combined by PCR using a temperature cycle profile of 94 °C for 3 min, 5 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min followed by 72 °C for 10 min. The final full-length clones (∼2 kb, 1758 bp from start to stop codon) of *CtNH1* were obtained using the above combined 5′/3′ PCR product using GeneRacer 5′-primer/GeneRacer 3′-primer by touch-down PCR using a temperature cycle profile of 94 °C for 2 min, 5 cycles of 94 °C for 30 s, 72 °C for 2 min, 5 cycles of 94 °C for 30 s, 70 °C for 2 min, 5 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 2 min, and followed by 72 °C for 10 min. The PCR products were purified from gel and cloned into the TOPO vector pENTR™/D-TOPO using pENTR™ Directional TOPO cloning kits (Invitrogen, USA). The resultant constructs were verified by sequencing.

### 2.2. RNA extraction and assays

Total RNA was isolated from young leaves with Trizol Reagent, according to the manufacturer's protocol (Invitrogen, Carlsbad, CA).

For quantitative RT-PCR assays, 'Duncan' grapefruit plants were infiltrated with water or *Xac* strain 306 (*Xac*306), or sprayed with 0.5 mM SA solution. Bacterial suspensions at $OD_{600} = 0.3$ were used. Leaf samples were collected at 0, 6, 24, and 48 h after treatments. Total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Isolated RNA was treated with RNase-free DNase (Qiagen, Valencia, CA) to eliminate genomic DNA contamination prior to cDNA synthesis. Reverse transcription was performed using GoScript™ Reverse Transcription System (Promega, Madison, WI) according to the manufacturer's instruction.

PCR amplification was performed in a 20 μl reaction containing 1 μl of diluted cDNA template, 100 nM of each primer, and 10 μl of Absolute Blue qPCR SYBR Green mix (Thermo Fisher Scientific, Surrey, UK) under the following conditions: 15 min at 95 °C followed by 40 cycles of 15 s at 95 °C, 15 s at 60 °C, and 15 s at 72 °C. Three biological replicates for each sample were performed. Data analyses were performed according to the manufacturer's instructions. Results were normalized to the expression of EF1α RNA.

For RNA blot assays, each 10 μg of total RNA was separated in 1.0% formaldehyde gel and transferred to IMMOBILON-NY + membranes (Millipore, Billerica, MA) for RNA blot analyses. The full-length *CtNH1* cDNA described above was labeled with [α-$^{32}$P]dCTP using a random primer labeling kit (Stratagene, Cedar Creek, TX) for hybridization and autoradiography.

The *chitinase 1* (*Chi1*)-specific probe was RT-PCR amplified from pummelo using PrimerChi1-1/PrimerChi1-2, verified by DNA sequencing and subjected to RNA blot analyses.

### 2.3. Sequence analyses

Sequences were aligned using CLUSTAL W [33], and the phylogenetic tree was generated by the neighbor-joining method [34] using the MEGA4 program [35].

### 2.4. Construction of citrus transformation plasmid

To engineer the *EcoRV* sites at both ends, the cloned *CtNH1* gene was PCR amplified using PrimerCtNH1-1/PrimerCtNH1-2 (Suppl. Table 1). After gel purification, the PCR product was then cloned into the *SmaI* site of the binary vector pTLAB31. The resultant construct was verified by sequencing.

### 2.5. Transformation and transgenic plants survey

Agrobacterium-mediated transformation of 'Duncan' grapefruit was performed as previously described [36]. The explants obtained from citrus seedlings germinated *in vitro* were used in co-incubation experiments. After regeneration, putative transgenic shoots were micro grafted *in vitro* onto 'Carrizo' citrange [*Citrus sinensis* (L.) Osb. × *Poncirus trifoliata* (L.) Raf.] rootstock and then transferred to soil when reaching about 4 cm in height.

Once the grafted seedlings produced their first well developed leaves, genomic DNA was isolated from the seedlings using GenElute Plant Genomic DNA Miniprep kit (Sigma–Aldrich, St Louis, MO) according to the manufacturer's instructions and subjected to PCR analysis to confirm the transgenic identity of the individual plants. The primers used for the PCR reaction include PrimerCtNH1-3/PrimerCtNH1-4 (Suppl. Table 1).

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CtNH1      MDNRNG-FSDSNEISNNSRTSCVAAAANTESFYSSEPVNS-DITALRILSKTLETIFESQDFD-YFTDAKIVL   70
TcNPR1     MDNRNG-FSDSNEISNNSSTCCIAAATNSETLASSEPLNTPDIAAALQILSRNLESVFESTDSDSLYSDAKIGL   72
VvNPR1.1   MDYRAA-LSDSNDFSGSSSICCIAATT--ESLSS--EVSPPDISALRRLSENLESVFESPEFD-FFTDARIVV   67
OsNPR1     MEPPTS-HVT-NAFSDSDSASVEEGGAD----------ADADVEALRRLSDNLAAAFRSPEDFAFALADARIAV   61
AtNPR1     MDTTIDGFADSYEISSTSFVATDNTDSSIVYLAAEQVLTGPDVSALQLLSNSFESVFDSPDDF--YSDAKLVL   71

CtNH1      STGR----EVPVHRCILSSRSDFFKNVFAGT--------GKQRGPKFELKELVR----DYEVGFDPLVAVLAY  127
TcNPR1     SSGR----EVPVHRCILSARSSVFKTVFSG---------LKDRGAKFELKELAR----DYEIGYDSLVAVLAY  128
VvNPR1.1   AGGR----EVPVHRCILAARSVFFKAVLAGA--------RKEKEAKFELKDLAK----EFDVGYDSLVAVLGY  124
OsNPR1     PGGGGGGGDLLVHRCVLSARSPFLRGVFARRAAAAAGGGGEDGGERLELRELLGGGGEEVEVGYEALRLVLDY  134
AtNPR1     SDGR----EVSFHRCVLSARSSFFKSALAAAKKEK--DSNNTAAVKLELKEIAK----DYEVGFDSVVTVLAY  134
                                            BTB domain

CtNH1      LYCGKVRPFPIGVCVCVDDDACSHVACRPAVDFMVEVLYVSFAFQVPELVALYQRHLLDILDKVVADDILVVL  200
TcNPR1     LYSGRVRSLPRGVCVCVDDD-CSHLACRPAVDFVAEVLYAAFTFQVSELISLYQRHLLDIIDKVEMDDILVVL  200
VvNPR1.1   LYSGRVGALPKGVCACVDDD-CPHSACRPAVDFMVEVLYASFAFQISELVGLYQRRLMDILDKVASDDILVIL  196
OsNPR1     LYSGRVGDLPKAACLCVDED-CAHVGCHPAVAFMAQVLFAASTFQVAELTNLFQRLLDVLDKVEVDNLLLIL  206
AtNPR1     VYSSRVRPPKGVSECADEN-CCHVACRPAVDFMLEVLYLAFIFKIPELITLYQRHLLDVVDKVVIEDTLVIL  206

CtNH1      SVAHMCGKACEKLLERCIEITVKSDIDIVTLDKTLPQHIVKQIIDLRVELSLHRSESCGFPDKHTKRIHRALD  273
TcNPR1     YVANMCGNTCERLLAKCIETLVKSDVDIVTLDKALPYHIVKQIMDSRLELGLDKPENTGFPDKHVKRIHRALD  273
VvNPR1.1   SVANLCGKACDRLLARCIDIIIKSDVDVVTLERALPQEMVKQIVDSRLELGFEEPESTNFPDKHVKRIHRALD  269
OsNPR1     SVANLCNKSCMKLLERCLDMVVRSNLDMITLEKSLPPDVIKQIIDARLSLGLISPENKGFPNKHVRRIHRALD  279
AtNPR1     KLANICGKACMKLLDRCKEIIVKSNVDMVSLEKSLPEELVKEIIDRRKELGLEVPKVK----KHVSNVHKALD  275

CtNH1      SDDVELVRMLLKEAHTNLDDAHALHYAVAYCDAKTTTELLDLGLADVNHRNSRGHTVLHVAAMRKEPKIIVSL  346
TcNPR1     SDDVELARMLLKEGHTNLDEASALHYAVAYCDAKTTTELLDLGLADVNRRNSRGYTVLHVAAMRKEPKIIVSL  346
VvNPR1.1   SDDVELVRMLLKEGHTTLDDAYALHYAVAFGDAKTTTELLDLGLADVNHKNHRGYTVLHIAAMRKEPKIIVSL  342
OsNPR1     SDDVELVRMLLTEGQTNLDDAFALHYAVEHCDSKITTELLDLALADVNHRNPRGYTVLHIAARRREPKIIVSL  352
AtNPR1     SDDIELVKLLLKEDHTNLDDACALHFAVAYCNVKTATDLLKLDLADVNHRNPRGYTVLHVAAMRKEPQLILSL  348
                                            Ankyrin domain

CtNH1      LTKGARPSDLTLDGRKALQISKRLTKAADYYIPTEEGKTTPKDRLCIEILEQAERRDPLLREASHSFAMAGDD  419
TcNPR1     LTKGARPSDLTLDGRKAFQISKRLTRAADYYMSTEEGKASPKDRLCVEILEQAERRDPLLGEASLSLAMAGDD  419
VvNPR1.1   LTKGARPTDITPDGRNALQIAKRLTRAVDYHKSTEEGKPSPKDQLCVEVLEQAERRDPLIGEASFSLAIAGDD  415
OsNPR1     LTKGARPADVTFDGRKAVQISKRLTKQGDYFGVTEEGKPSPKDRLCIEILEQAERRDPQLGEASVSLAMAGES  425
AtNPR1     LEKGASASEATLEGRTALMIAKQATMAVECNNIPEQCKHSLKGRLCVEILEQEDKREQIPRDVPPSFAVAADE  421

CtNH1      LRMKLLYLENRVGLAKLLVPMEAKVIMDIVHLDGTLEFTLDGIKTKKMAGAQRTTVDLNEAPFKMQEEHLSRM  492
TcNPR1     LRMKLLYLENRVGLAKLLFPMEAKVAMDIAKVDGTSEFTLASINSNKLNDAQRTTVDLNEAPFRIQEEHLNRL  492
VvNPR1.1   LRMKLLYLENRVGLAKLLFPMEAKVAMDIAQVDGTSEFTLTAIRPRNLADAQRTTVDLNEAPFRIKEEHLNRL  488
OsNPR1     LRGRLLYLENRVALARIMFPMEARVAMDIAQVDGTLEFNLGSG-ANPPPERQRTTVDLNESPFIMKEEHLARM  497
AtNPR1     LKMTLLDLENRVALAQRLFPTEAQAAMEIAEMKGTCEFIVTSLEPDRLTGTKRTSPGVKIAPFRILEEHQSRL  494

CtNH1      KALCRTVELGKRFFPRCSEVLNKIMDADDLNQLACPGNDTPEERLLKRIRYMELQEVVSKAFNEDKEEFDRSA  565
TcNPR1     KALSRTVELGKRFFPRCSEVLNKIMDADDLSQLACGGNDTPEERLVKKQRYVELQDVLSKAFNEDKVEFDRST  565
VvNPR1.1   RALSKTVDLGKRFFPRCSEVLNKIMDADDLSDLAYLGNGTTEERLLKKRRYKELQDQLCKAFNEDKEENDKSR  561
OsNPR1     TALSKTVELGKRFFPRCSNVLDKIMD--DETDPVSLGRDTSAE---KRKRFHDLQDVLQKAFHEDKEENDRSG  565
AtNPR1     KALSKTVELGKRFFPRCSAVLDQIMNCEDLTQLACGEDDTAEKRLQKKQRYMEIQETLKKAFSEDNLELGNSS  567
                                                                  NLS

CtNH1      ISSSSSSK--SVVRPR-GGKRTH      585
TcNPR1     ISSSSSSK--SIGVSRPNGKLTGSGRGG  591
VvNPR1.1   ISSSSSST--SLGFGRNNSRLSCKK    584
OsNPR1     LSSSSSST--SIGAIRPRR          582
AtNPR1     LTDSTSSTSKSTGGKRSNRKLSHRRR   593
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**Fig. 1.** Multiple sequence alignment of CtNH1 with Arabidopsis NPR1 (AtNPR1) and its orthologs from rice (OsNPR1/NH1), grapevine (VvNPR1.1), and cacao (TcNPR1). Residues conserved among the NPR1 proteins are highlighted in bold. Amino acids in CtNH1 identical to the NPR1 conserved residues are also in bold. The BTB/POZ and ankyrin domains and the bipartite nuclear localization signal (NLS) of AtNPR1 are indicated, whereas the functionally critical Cys82 and Cys216 are shown in larger fonts. Dashed lines represent gaps introduced for maximum alignment. Accession numbers for the used sequences are listed in the legend of Fig. 2.

### 2.6. Bacterial preparation and inoculation procedures

Wild-type *Xac*306 was cultured on a standard nutrient agar medium (Difco, Detroit, MI) for two days at 28 °C. The bacterial suspension at a concentration of $10^5$ CFU/ml was used for infiltration into the abaxial surface of the leaves, using a needleless syringe. Disease symptoms were photographed and scored two-weeks after inoculation. For planta-growth assays, leaf disks were excised with a cork borer (leaf area: 1 cm$^2$) and then ground in 1 ml sterile tap water. The samples were serially diluted and plated on

NA plates supplemented with the antibiotic rifampicin, and colonies were counted 48 h after plating. The tests were repeated three times with similar results.

## 3. Results

### 3.1. Isolation and characterization of CtNH1

To isolate a *NPR1* homolog from citrus, RT-PCR was performed using RNA isolated from pummelo as the template. A full-length

cDNA containing a 1758 bp open reading frame (ORF) was obtained. This ORF was named *CtNH1*.

*CtNH1* encodes a 586 amino acid protein (CtNH1) containing a BTB domain at the N-terminal half, four ankyrin repeats in the central region, and a bipartite nuclear localization signal (NLS) at the C-terminal portion (Fig. 1). Alignment of CtNH1 to AtNPR1 and three known NPR1 orthologs from rice, grapevine, and cacao indicates that the above domains are conserved among all these proteins (Fig. 1). In addition, a total of 213 residues, including the cysteine residues Cys82 and Cys216 important for the oligomerization of AtNPR1 [12], are identical among the NPR1 orthologs (Fig. 1 and Table 1). Most of the NPR1 conserved amino acid residues (94.4%) are also present in CtNH1 (Fig. 1 and Table 1). Further sequence comparisons reveal that CtNH1 shares high levels of similarity with AtNPR1 (73% similarity, 54% identity), OsNPR1/NH1 (72% similarity, 58% identity), VvNPR1.1 (83% similarity, 71% identity), and TcNPR1 (87% similarity, 78% identity).

To test whether *CtNH1* is induced by *Xac* infection or SA application, we carried out quantitative RT-PCR assays. As shown in Suppl. Fig. 1, RNA transcripts of *CtNH1* are not significantly altered by the treatments, indicating that *CtNH1* may not be regulated at the transcription levels by pathogens or the SAR inducers.

### 3.2. Phylogenetic analysis of NPR1-related gene products

Phylogenetic analysis was performed to elucidate the history of CtNH1. In the sequenced sweet orange (*C. sinensis*) genome, there are six genes whose predicted products are similar to AtNPR1 or its paralogs. CtNH1 is almost identical to organe1.1g007923m at the amino acid level with only five residue variations. Both CtNH1 and organe1.1g007923m belong to the clade that consists of functional AtNPR1, OsNPR1/NH1, VvNPR1.1, and TcNPR1, suggesting that they have all evolved from a common ancestor (Fig. 2). Within this clade, AtNPR2 is the most closely-related to AtNPR1. However, multiple

sequence alignment indicates that only 81.2% (173 out of 213) of the NPR1 conserved residues are retained in AtNPR2 and the residue corresponding to the functionally critical Cys216 of AtNPR1 is replaced with tyrosine in AtNPR2 (Table 1). Therefore, AtNPR2 might have a function differing from AtNPR1. Interestingly, AtNPR2 interacts with AtNPR1 in yeast [18]. However, the biological function of this interaction remains to be elucidated.

Consistent with their biological roles, AtNPR3-4 and AtBOP1-2 form two clades distinct from the AtNPR1 group in the phylogenetic tree (Fig. 2). Interestingly, MpNPR1-1 is a member of the AtNPR3/AtNPR4 group that also contains VvNPR1.2 from grapevine. Expression of the *MpNPR1* gene is induced by BTH treatment and over-expression of *MpNPR1* in apple confers enhanced resistance [28]. It has been suggested that *MpNPR1* could be an ortholog of *AtNPR1*. Similar to *MpNPR1-1*, *VvNPR1.2* expression is induced by BTH, however this gene was unable to complement the Arabidopsis *npr1-2* mutant [31,32]. In contrast, *VvNPR1.1* from the same genome complements the mutant [32]. These observations suggest that the members of the AtNPR3/AtNPR4 group may not be true orthologs of *AtNPR1*. Indeed, recent studies indicate that AtNPR3 and AtNPR4 act as both SA receptors and adapters, linking NPR1 with the CUL3 E3 ligase [18].

### 3.3. Over-expression of CtNH1 in citrus

To functionally characterize *CtNH1*, the full-length cDNA of this gene was cloned into the binary vector pTLAB31 under the control of the *Figwort mosaic virus* (FMV) promoter. The resultant construct was then introduced into *Agrobacterium tumefaciens* for transforming the susceptible citrus cultivar 'Duncan' grapefruit (*Citrus paradisi* L.). To obtain multiple transgenic lines, citrus transformation was conducted several times on different dates. A total of 8500 explants of 'Duncan' grapefruit were used and fifteen lines were obtained. PCR assays indicated that five were positive for

**Table 1**
An overview of multiple sequence alignment of NPR1-related proteins.

| Name | Subfamily | Identity to NPR1 conserved residues | Residues corresponding to Cys82 of AtNPR1 | Residues corresponding to Cys216 of AtNPR1 |
|---|---|---|---|---|
| CtNH1 (pummelo) | AtNPR1 | 94.4% (201/213) | C | C |
| Orange1.1g007923m (sweet orange) | | 94.8% (202/213) | C | C |
| TcNPR1 (cacao) | | 100% (213/213) | C | C |
| GhNPR1 (cotton) | | 93.9% (200/213) | W | C |
| CpNPR1 (papaya) | | 68.5% (146/213) | C | C |
| LeNPR1 (tomato) | | 93.0% (198/213) | C | C |
| NtNPR1 (tobacco) | | 94.4% (201/213) | C | C |
| CaNPR1 (chili pepper) | | 94.4% (201/213) | C | C |
| VvNPR1.1 (grapevine) | | 100% (213/213) | C | C |
| OsNPR1/NH1 (rice) | | 100% (213/213) | C | C |
| MNPR1A (banana) | | 92.0% (196/213) | C | C |
| MNPR1B (banana) | | 91.5% (195/213) | C | C |
| AtNPR1 (Arabidopsis) | | 100% (213/213) | C | C |
| AtNPR2 (Arabidopsis) | | 81.2% (173/213) | C | Y |
| NH3 (rice) | AtNPR3/4 | 73.2% (156/213) | C | L |
| NH2 (rice) | | 70.4% (150/213) | C | L |
| Orange1.1g007758m (sweet orange) | | 54.9% (117/213) | S | L |
| Orange1.1g008204m (sweet orange) | | 53.1% (113/213) | S | L |
| Orange1.1g007550m (sweet orange) | | 67.1% (143/213) | C | L |
| AtNPR3 (Arabidopsis) | | 65.3% (139/213) | C | L |
| AtNPR4 (Arabidopsis) | | 67.1% (143/213) | C | L |
| Orange1.1g009554m (sweet orange) | | 62.4% (133/213) | C | L |
| VvNPR1.2 (grapevine) | | 71.4% (152/213) | C | S |
| MpNPR1-1 (apple) | | 67.6% (144/213) | C | L |
| NH5.1 (rice) | AtBOP1/2 | 63.8% (136/213) | C | M |
| NH5.2 (rice) | | 63.8% (136/213) | C | M |
| NH4 (rice) | | 61.0% (130/213) | C | L |
| AtBOP1 (Arabidopsis) | | 39.0% (83/213) | C | M |
| AtBOP2 (Arabidopsis) | | 39.4% (84/213) | C | M |
| Orange1.1g010590m (sweet orange) | | 41.3% (88/213) | C | M |
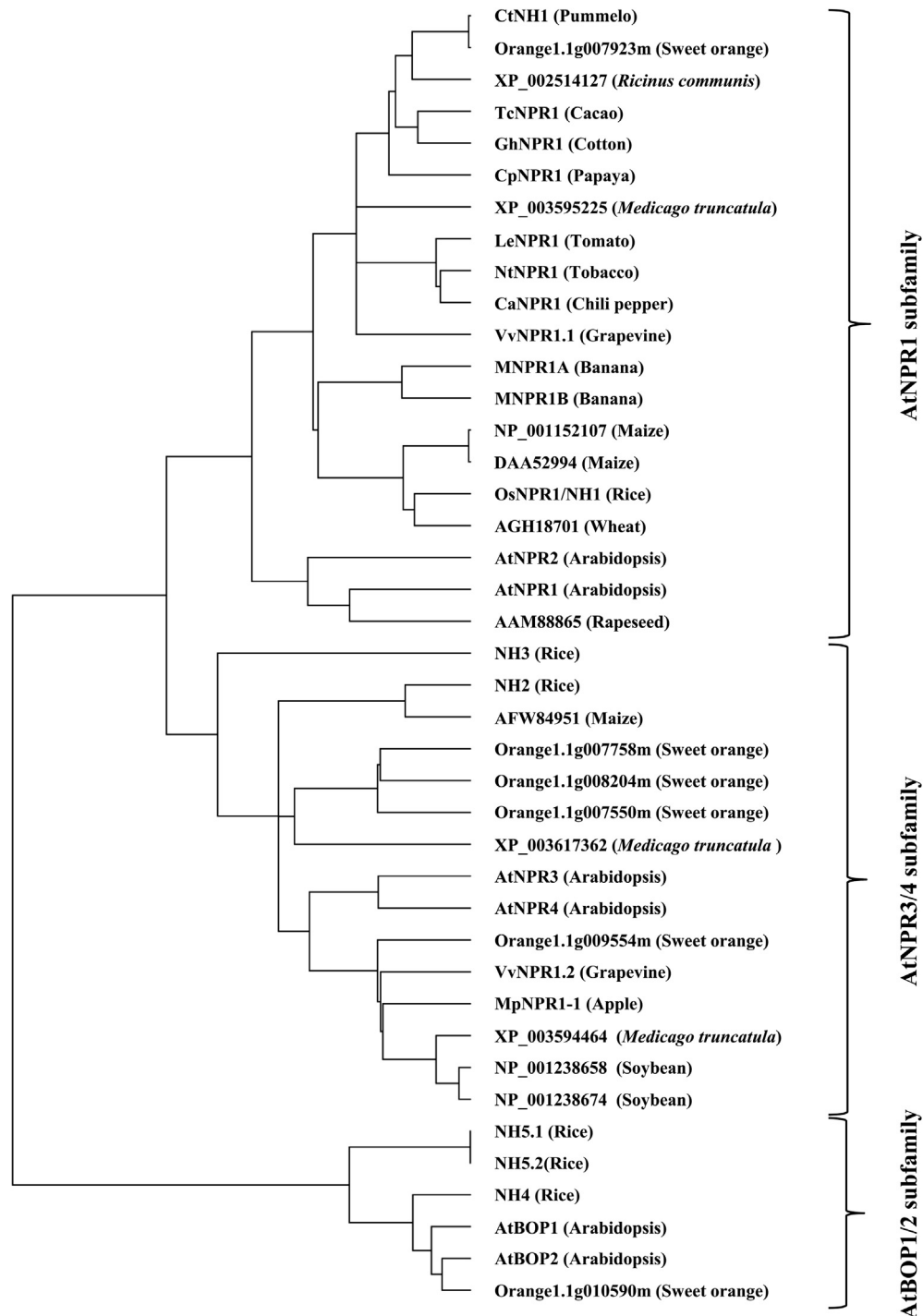
**Fig. 2.** Phylogenetic analyses of NPR1 orthologs and homologs from different plant species. Accession numbers for sequences of the NPR1-related proteins are AtNPR1: At1g64280 (Arabidopsis), AtNPR2: At4g26120 (Arabidopsis), AtNPR3: At5g45110 (Arabidopsis), AtNPR4: At4g19660 (Arabidopsis), AtBOP1: At3g57130 (Arabidopsis), AtBOP2: At2g41370 (Arabidopsis), OsNPR1/NH1: Os01g09800 (rice), NH2: Os01g56200 (rice), NH3: Os03g46440 (rice), NH4: Os01g72020 (rice), NH5.1: Os11g04600 (rice), NH5.2: Os12g04410 (rice), VvNPR1.1: GSVIVP00016536001 (grapevine), VvNPR1.2: GSVIVP00031933001 (grapevine), TcNPR1: HM117159 (cacao), LeNPR1: GI49182274 (tomato), CpNPR1: GI45331147 (papaya), MpNPR1-1: ACC77697.1 (apple), NtNPR1: GI111054505 (tobacco), CaNPR1: GI109693029 (chili pepper), MNPR1A: DQ925843 (banana), MNPR1B: EF137717 (banana), GhNPR1: ABV68572 (cotton). Accession numbers of other NPR1 homologs are indicated.

CtNH1 (data not shown). Two of the five confirmed transgenic plants appeared significantly smaller in size at the time of the assays due to late regeneration. The two younger lines eventually reached normal size. Since we had already selected three lines, CN1-1, CN1-3, and CN1-5, for further characterization, these plants were not further propagated by grafting, nor subsequently inoculated and characterized.

The transcript levels of *CtNH1* in these transgenic lines were determined by RNA blot analyses. All three transgenic lines accumulated high levels of transcripts (Fig. 3). The majority of the
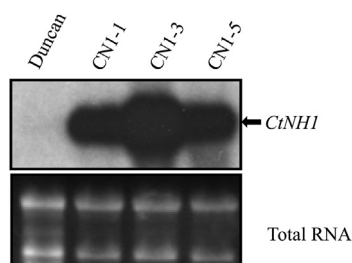
**Fig. 3.** RNA blot analyses showing *CtNH1* transcript levels in the indicated transgenic lines and the recipient line 'Duncan' grapefruit. Total RNA isolated from leaf tissues was probed with a *CtNH1*-specific sequence. Both autorad (*upper*) and ethidium bromide-stained agarose gel (*lower*) are shown.

observed *CtNH1* transcripts should be produced from the transgene, because no detectable *CtNH1* was seen in the recipient line 'Duncan' grapefruit. Among the three lines, CN1-3 showed the highest level of *CtNH1* transcripts.

### 3.4. Resistance to citrus canker

The three transgenic lines with high levels of *CtNH1* transcripts were inoculated with *Xac306* to determine whether over-expression of this gene confers resistance to citrus canker. Plants were inoculated by leaf infiltration and disease symptoms were scored 14 days after inoculation. All three *CtNH1* over-expression lines showed brownish lesions, but developed significantly less severe callus-like lesions (canker symptoms) as compared with 'Duncan' grapefruit plants (Fig. 4). The development of brownish lesions in the *CtNH1* over-expression lines might be associated with the inoculation method and cell death phenotypes were also observed in some *AtNPR1* over-expression lines [25]. By contrast, we did not observe a similar tissue collapse when the plants were spray-inoculated with *Xac306* inoculum (data not shown). Compared to the leaf spraying method, leaf infiltration is, however, a more reliable means to initiate disease development on 'Duncan' grapefruit plants. Therefore, leaf infiltration was used as the primary inoculation technique in this study.

To confirm the observed resistance, we carried out growth curve analyses to compare bacterial multiplication in CN1-1 and 'Duncan' grapefruit plants. After inoculation by infiltration with a suspension of *Xac306*, leaf samples of CN1-1 and 'Duncan' grapefruit were harvested from the infiltrated area at different time intervals. Consistent with the lesion development data described above, the *Xac* population is differentiated in the infiltrated leaves of CN1-1 and 'Duncan' grapefruit as early as five days after inoculation (DAI). At 19 DAI, the level of *Xac306* in CN1-1 plants is $10^4$ fold lower than that in 'Duncan' grapefruit. Taken together, these results indicate that over-expression of *CtNH1* results in a high level of resistance to *Xac306*.

### 3.5. Constitutive expression of the Chi1

Over-expression of *AtNPR1* or its orthologs is often associated with a constitutive expression of some *PR* genes [23,26]. Because induction of *Chi1* has been clearly correlated with a resistance response in citrus [37], we therefore use *Chi1* levels as an indicator of the citrus defense system. RNA blot analyses revealed that *Chi1* transcripts are drastically increased in all three *CtNH1* over-expression lines as compared with an undetectable basal level of this gene in wild-type plants (Fig. 5). These results indicate that *Chi1* is constitutively expressed in the *CtNH1* over-expression lines
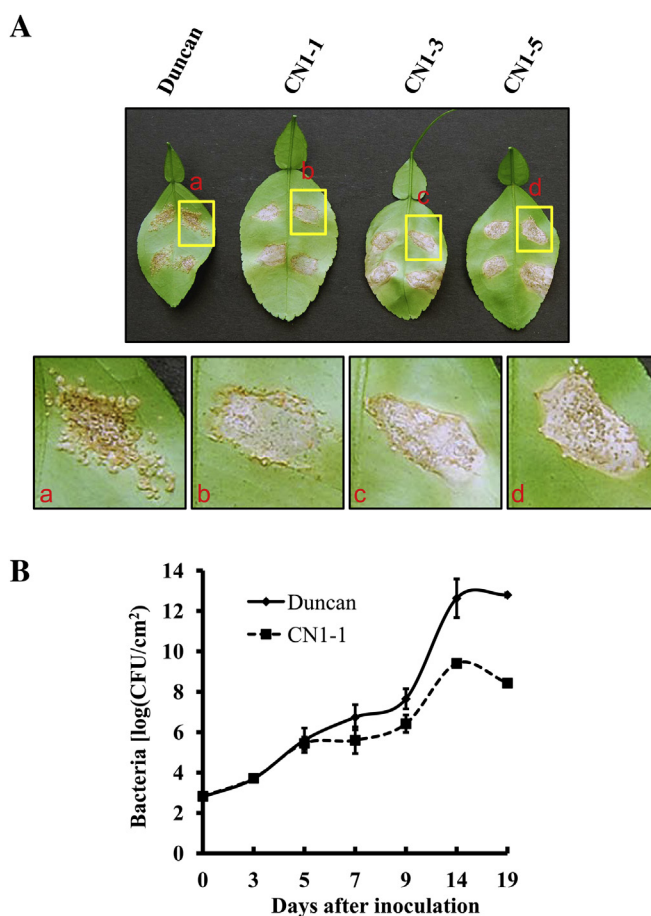


**Fig. 4.** Transgenic plants over-expressing *CtNH1* display enhanced resistance to *Xac306*. (A) Photograph of inoculated leaves showing disease symptoms two weeks after inoculation with *Xac306*. Close-up images of indicated areas are shown below. Raised pustules, a typical symptom for citrus canker diseases, are evident only on the susceptible control line 'Duncan' grapefruit. (B) Growth of *Xac306* in the transgenic line CN1-1 (dashed line) and the control 'Duncan' grapefruit (solid line). Bars represent standard error of the mean.

and suggest that over-expression of *CtNH1* leads to an activation of defense in citrus in the absence of pathogens.

## 4. Discussion

Isolation and characterization were performed on a citrus homolog of *AtNPR1*. Multiple lines of evidence suggest that *CtNH1* is the citrus ortholog of *AtNPR1*. First, the predicted product of *CtNH1* contains all functional domains of AtNPR1 that include a BTB domain, four ankyrin repeats, and an NLS. Second, there are 213 conserved residues, including the cysteine residues (Cys82 and Cys216 in Arabidopsis) critical for NPR1 oligomerization, among AtNPR1 and its known orthologs (OsNPR1/NH1, VvNPR1.1, and TcNPR1). Our sequence analysis indicates that 94.4% (201 out of 213) of the residues are also conserved in CtNH1 (Table 1). In contrast, AtNPR2 only contains 81.8% (175 out of 213) of the conserved residues. Third, CtNH1 is phylogenically-related to AtNPR1 and its orthologs, but distinct from other AtNPR1 paralogs, except for AtNPR2. Fourth, similar to *AtNPR1* [25], over-expression of *CtNH1* in citrus results in resistance to *Xac*.

A combination of phylogenetic analyses and multiple sequence alignment may be a better strategy to predict functional NPR1 orthologs in plant species. In addition to CtNH1 and orange1.1g007923m, we predict that MNPR1A/B, NtNPR1, CaNPR1,
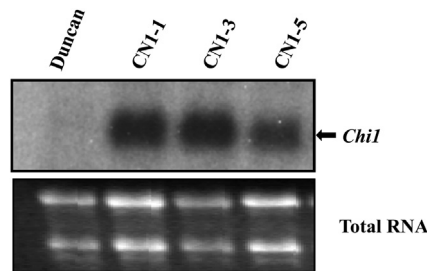
**Fig. 5.** Constitutive expression of the *PR* gene *Chi1* in *CtNH1* over-expression lines. RNA blot analyses showing *CtNH1* transcript levels in the indicated transgenic lines and the recipient line 'Duncan' grapefruit. Total RNA isolated from leaf tissues was probed with a *Chi1*-specific sequence. Both autorad (*upper*) and ethidium bromide-stained agarose gel (*lower*) are shown.

and LeNPR1 from banana, tobacco, chili pepper, and tomato, respectively, are orthologous to AtNPR1 (Fig. 2 and Table 1). Notably, the banana genome may contain two NPR1 orthologs. GhNPR1 from cotton belongs to the NPR1 clad in the phylogenetic tree, however the critical Cys82 has been mutated to tryptophan, suggesting that this gene might not be functional. The papaya CpNPR1 is also a member of the NPR1 clad and contains cysteine residues in the positions corresponding to 82 and 218 of AtNPR1. Because of a relative low percentage identity (68.5%) among the 213 NPR1 conserved residues, this gene might not be a true ortholog.

Based on the information from sequenced genomes, the AtNPR1 subfamily appears to be more conserved than the AtNPR3/4 subfamily. For example, the sweet orange and rice genomes all contain a single gene coding for counterparts of AtNPR1 (Fig. 2). By contrast, there are four members (Orange1.1g009554m, Orange1.1g007758m, Orange1.1g008204m, and Orange1.1g007550m) of the AtNPR3/4 subfamily in sweet orange. Among them, Orange1.1g009554m is most related to AtNPR3 and AtNPR4, whereas the other three proteins form a unique subclade. Given that AtNPR3 and AtNPR4 act as SA receptors, it would be interesting to investigate whether all of the four sweet orange proteins are involved in SA perception or if only Orange1.1g009554m functions as the SA receptor and the other three proteins link NPR1 to other stimuli.

Our study indicates that *CtNH1* transcripts in wild-type plants accumulate to a level undetectable by RNA blot analyses in total RNA (Fig. 3). When over-expressed in citrus, *CtNH1*, like *AtNPR1*, confers resistance to *Xac*. This is consistent with the notion that the *NPR1* signaling is conserved. Different from the *AtNPR1* over-expression experiments [25], we used the FMV promoter, rather than the *Cauliflower mosaic virus* (CaMV) 35S promoter, to drive *CtNH1*. Previous studies in soybean have showed that the FMV promoter is significantly stronger than the widely used 35S promoter for gene expression [38]. Therefore, our transgenic lines may represent plants with higher levels of transgenes.

In the characterized *CtNH1* plants, we found constitutive expression of the *PR* gene *Chi1*. Induction of this gene has been found in citrus plants treated with various stimuli, including UV irradiation, wounding, and the treatment with β-aminobutyric acid or *Candida oleophila* yeast cells, all of which can trigger a resistance response [37]. Therefore, a defense response is likely activated constitutively in the *CtNH1* over-expression lines in the absence of a SAR inducer. This observation differs from the results from Arabidopsis plants over-expressing *AtNPR1*, where *PR* gene induction depends on a SAR inducer or pathogen infection [19,20]. However, our result is in line with the observations made from transgenic tomato and rice plants over-expressing *AtNPR1* and *OsNPR1/NH1*, respectively [23,26]. There is an apparent difference when *AtNPR1* is expressed in specific plant species. The molecular details of this

discrepancy are unknown. A possible mechanism is that higher levels of NPR1 might lead to partial activation of this protein in certain species. It has also been reported that over-expression of *OsNPR1/NH1* results in a lesion-mimic phenotype [26]. Despite *Chi1* expression, we did not observe obvious detrimental morphological phenotypes in the citrus *CtNH1* over-expression lines. Thus, *NPR1* might have distinct effects on cell signaling when over-expressed in different plant species.

The lack of resistant sources to major diseases is a significant challenge for citrus protection. Despite the long history of breeding, varieties resistant to citrus canker remain to be developed. Meanwhile, serious disease threats posed by new and emerging citrus pathogens have appeared. Our work indicates that targeted gene approaches based on the information from model plant species can significantly expedite the process of identifying resistance genes/regulators from citrus. *NPR1* is crucial for SAR and increasing evidence suggests that the NPR1 signaling is conserved across monocotyledonous and dicotyledonous plants. Therefore, *CtNH1* has a potential to serve as an important source for broad resistance to citrus pathogens.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.pmpp.2013.07.002.

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