Genes & Genomics (2012) 34: 379-390
DOI 10.1007/s13258-011-0218-7

RESEARCH ARTICLE

The *NPR1* family of transcription cofactors in papaya: insights into its structure, phylogeny and expression

Santy Peraza-Echeverria · Jorge M. Santamaría · Gabriela Fuentes · Mariana de los Ángeles Menéndez-Cerón · Miguel Ángel Vallejo-Reyna · Virginia Aurora Herrera-Valencia

Received: 08 November 2011 / Accepted: 12 January 2012 / Published online: 25 June 2012

Abstract
The *NPR1* (non-expressor of pathogenesis related gene 1) gene was initially identified in *Arabidopsis* as a master regulator of the systemic acquired resistance (SAR). Five additional *NPR1* homologues have been identified in *Arabidopsis* whose function range from regulators of SAR to plant development. In the present study, we characterized the structure, phylogeny and expression of the *NPR1* family in papaya (*Carica papaya* L.), one of the most important tropical fruit crops. We identified four *NPR1* homologues in the papaya genome sequence (*CpNPR1* to *CpNPR4*). Overall, the four papaya predicted NPR1 proteins showed the characteristic BTB/POZ and ankyrin domains of the *Arabidopsis* NPR1 family. Twelve additional open reading frames showing homology to retrotransposon elements or genes involved in different physiological processes were found in close proximity to the papaya *NPR1* homologues. The phylogenetic analysis revealed that the papaya NPR1 sequences resolved in three clades, each clade containing two *Arabidopsis* NPR1 homologues involved either in the positive regulation of SAR (clade I), negative regulation of SAR (clade II) or plant development (clade III), suggesting a similar function for the corresponding papaya NPR1 homologues. Furthermore, the expression of the four papaya *NPR1* homologues was detected in both vegetative and reproductive tissues. The present study has provided the first comparative analysis of the *NPR1* family in a tropical fruit crop and expanded our knowledge on this type of genes in dicotyledoneous plants. The identification of the full set of papaya *NPR1* homologues will pave the way for their systematic functional analysis and new opportunities for engineering disease resistance in this crop.

Keywords Papaya; *NPR1* family; BTB/POZ; Ankyrin; SAR; Development

S. Peraza-Echeverria (✉) · J. M. Santamaría · G. Fuentes · M. de los Á. Menéndez-Cerón · M. Á. Vallejo-Reyna · V. A. Herrera-Valencia
Unidad de Biotecnología, Centro de Investigación Científica de Yucatán (CICY), C. 43, No. 130, Col. Chuburná de Hidalgo, Mérida, Yucatán, Postal Code 97200, México.
e-mail: santype@cicy.mx

Introduction

Papaya (*Carica papaya* L.), is one of the major fruit crops cultivated in the tropics and sub-tropics. Papaya fruit is known for its high nutritive and medicine value. Total annual world production of papaya is estimated over 10.4 million tonnes, and the countries that dominate the production and exportation of this important commodity are India and Mexico, respectively (FAOSTAT, 2011). Diseases caused by virus, bacteria, fungi and nematodes are major constraints in the production of this crop (American Phytopathological Society, 2011). In this context, the identification of disease resistance-related sequences from the papaya genome has the potential to assist in the development of new disease resistant varieties. The sequencing of the complete papaya genome (Ming et al., 2008) has paved the way to characterize the full set of papaya genes involved in disease resistance. Recently, the *NBS-LRR* family of disease resistance (R) genes and the *TGA* family of transcription factors were characterized *in silico* in this crop (Porter et al., 2009; Idrovo-Espín et al., 2012).

Systemic acquired resistance (SAR) is a broad-spectrum disease resistance response that involves the up regulation of a battery of pathogenesis-related (PR) genes (Durrant and Dong, 2004). Due to long-lasting and broad spectrum resistance against pathogens, employment of SAR for engineering disease resistance in plants is quite attractive. The *Arabidopsis NPR1* (*AtNPR1*) gene is considered as a master regulator of the SAR pathway given that the *Arabidopsis npr1* mutant that has lost its capacity to respond to salicylic acid (SA), cannot express PR genes and is unable to resist the attack of a broad spectrum of pathogens (Cao et al., 1994, 1997). In addition,

NPR1 is also involved in jasmonic acid (JA)/ethylene (ET)-dependent induced systemic resistance (ISR) triggered by some *Pseudomonas flourescens* strains, and has been shown to modulate cross talk between SA- and JA-dependent defense pathways (Lavicoli et al., 2003; Dong, 2004; Pieterse and Van Loon, 2004). *NPR1* encodes for a transcription cofactor protein containing two domains involved in mediating protein-protein interactions: the Broad Complex, Tramtrack and Bric a brac/Pox virus and Zinc finger (BTB/POZ) domain at the N-terminus and the ankyrin repeat domain in the central region of the protein (Cao et al., 1997; Hepworth et al., 2005; Spoel et al., 2009). In unchallenged plants, NPR1 resides in the cytoplasm as an oligomer maintained through redox-sensitive intermolecular disulfide bonds. Upon pathogen challenge, the plant defense signaling molecule SA increases and changes the cellular redox state, leading to reduction of the disulfide bonds in NPR1 and consequently the dissociation of the NPR1 oligomer into monomers (Mou et al., 2003). Recent data indicate that S-nitrosylation of NPR1 by S-nitrosoglutathione at cysteine-156 facilitates its oligomerization. Conversely, the SA-induced NPR1 oligomer-to-monomer reaction is catalyzed by thioredoxins (Tada et al., 2008). A bipartite nuclear localization sequence targets the released NPR1 monomer to the nucleus where it physically and differentially interacts with several members of the TGA family of basic domain/leucine zipper (bZIP) transcription factors (Zhang et al., 1999; Kinkema et al., 2000; Zhou et al., 2000). This NPR1-TGA interaction stimulates the activation of a battery of PR genes. In the absence of pathogen challenge, NPR1 is continuously cleared from the nucleus by the proteasome, which prevents activation of SAR. Accumulation of SA promotes NPR1 phosphorylation facilitating its recruitment to a Cullin3-based ubiquitin ligase. Turnover of phosphorylated NPR1 is required for full induction of target genes and establishment of SAR (Spoel et al., 2009).

In the *Arabidopsis* genome, *AtNPR1* belongs to a small gene family of five additional members (*AtNPR2, AtNPR3, AtNPR4, AtNPR5* and *AtNPR6*). Phylogenetic analysis of the *Arabidopsis* NPR1 protein family revealed three distinct groups (Hepworth et al., 2005; Zhang et al., 2006). AtNPR2 is the closest sequence to AtNPR1 and they form a pair; AtNPR3 and AtNPR4 form a second pair; and AtNPR5 and AtNPR6 form a third pair. Interestingly, AtNPR3 and AtNPR4 work as negative regulators of SAR (Zhang et al., 2006) while AtNPR5 and AtNPR6 function redundantly to control growth asymmetry in leaves and flowers (Hepworth et al., 2005), thus there is a correlation between the phylogenetic grouping pattern of these sequences and their function. The genome sequences of other plants such as *Oryza sativa* (Goff et al., 2002), *Populus trichocarpa* (Tuskan et al., 2006) and *Vitis vinifera* (Velasco et al., 2007), have also revealed a small number of *NPR1* homologues. Genes with high sequence similarity to *AtNPR1* have been reported in several crops including tobacco (Liu et al., 2002), papaya (Zhu et al., 2003), rice (Yuan et al., 2007), apple (Malnoy et al., 2007), cotton (Zhang et al., 2008), banana (Endah et al., 2008), grapevine (Le Henanff et al., 2009), soybean (Sandhu et al., 2009) and cacao (Shi et al., 2010).

Overexpression of *AtNPR1* in *Arabidopsis* (Cao et al., 1998; Friedrich et al., 2001), rice (Chern et al., 2001), tomato (Lin et al., 2004), wheat (Makandar et al., 2006), tobacco (Meur et al., 2008) and carrot (Wally et al., 2009) induced enhanced bacterial and fungal resistance. Likewise, overexpression of an endogenous *NPR1* orthologue in apple has led to enhance bacterial and fungal resistance (Malnoy et al., 2007). These findings make *NPR1* an attractive candidate for engineering disease resistance in tropical plants such as papaya. In the case of this crop, only one *NPR1* homologue has been reported so far (Zhu et al., 2003), therefore there is limited information about the total number of *NPR1* homologue genes in the papaya genome. The aims of the present study were to identify the full set of *NPR1* homologues from the papaya genome and to gain insights into their structure, phylogeny and expression. The new insights generated in the present study have provided a foundation for the in-depth systematic functional analysis of the papaya *NPR1* family members and potential applications for the genetic improvement of this crop.

Materials and Methods

Plant material

The genome sequence of the transgenic papaya cv. SunUp (Ming et al., 2008) was used to retrieve *NPR1* homologues. The papaya cv. Maradol was used in this study to evaluate the expression of *NPR1* homologues. This cultivar dominates the export trade of papaya in Mexico. The Maradol material was obtained from the commercial plantation Rancho Alegre, located in El Cuyo, Yucatán, México. Tissues of leaves, petioles, flowers and immature fruits were collected from healthy adult plants, immediately frozen in liquid nitrogen and stored at -80°C until use.

In silico cloning, annotation and protein structure homology modeling

The six members of the *Arabidopsis NPR1* family: *AtNPR1*, *AtNPR2*, *AtNPR3*, *AtNPR4*, *AtNPR5* and *AtNPR6*, with GenBank accession no. At1g64280, At4g26120, At5g45110, At4g19660, At2g41370 and At3g57130, respectively, were obtained from the *Arabidopsis* Information Resource (TAIR) (http://www.arabidopsis.org/). All these sequences were used as queries to search for *NPR1* homologues in the papaya cv. SunUp genome sequence. Similarity searches were performed in the Phytozome v7.0 database (http://www.phytozome.net/search.php) and GenBank (http://www.ncbi.nlm.nih.gov) using the BLASTP and TBLASTN algorithms (Altschul et al.,

1997). The open reading frames (ORFs) in the papaya DNA contigs harboring *NPR1* homologues were predicted by the FGENESH program with the *A. thaliana* genome set as reference (http://linux1.softberry.com/berry.phtml). The presence of BTB/POZ and ankyrin domains was detected using the SMART (http://smart.embl-heidelberg.de) (Letunic et al., 2009) and PROSITE (http://us.expasy.org/prosite) (Sigrist et al., 2010) databases. Percent amino acid identity between sequences was determined by the MegAlign program of the Lasergene software package version 7.2.1 (DNASTAR, Madison, WI). Homology modeling was performed on the SWISS-MODEL workspace server using the function of automatic mode (http://swissmodel.expasy.org) (Bordoli et al., 2009). The 3D models were visualized and edited with the RasMol program version 2.6 (http://rasmol.org).

Sequence alignment and phylogenetic analysis

Predicted protein sequences were aligned using the ClustalX program version 1.81 with the default settings (Thompson et al., 1997). Identical amino acids in the alignment were shaded with black color and conservative substitutions with gray color using the Boxshade program (http://www.ch.embnet.org). Phylogenetic trees were constructed by the neighbor-joining (NJ) method (Saitou and Nei, 1987) with the Poisson correction using the NJ algorithm implemented in the Molecular Evolutionary Genetic Analysis (MEGA) software version 5 (Tamura et al., 2011). Bootstrapping (1000 replicates) was used to evaluate the degree of support for a particular grouping pattern in the phylogenetic tree. All members of the *Arabidopsis* NPR1 family were used as queries to perform a BLASTP search in the GenBank in order to retrieve amino acid sequences of NPR1 homologues from different plant species for the phylogenetic tree construction. The six members of *Arabidopsis NPR1* family were also used as queries to perform BLAST searches in the microalga genome sequences of *Chlamydomonas reinhardtii*, *Chlorella variabilis* NC64A and *Volvox carteri* f. *nagariensis* (http://www.ncbi.nlm.nih.gov). Amino acids spanning from the start of BTB/POZ domain to the end of ankyrin domain were considered for the phylogenetic analysis.

Gene expression analysis by RT-PCR

Total RNA was extracted from leaves, petioles, flowers and immature fruits using a CTAB protocol described by Gasic et al. (2004). The concentration and purity of RNA samples were determined spectrophotometrically using a Nanodrop 1000 (Thermo Fisher Scientific Inc). Total RNA (4 μg) was treated with 1 U of RNase-free DNase (Promega) at 37°C for 15 min prior to cDNA synthesis. First-strand cDNA synthesis was carried out using 200 U of Superscript III reverse transcriptase (Invitrogen) according to manufacturer instructions. PCR mixtures (50 μℓ) contained 200 μM of dNTPs, 0.3 μM of each forward and reverse primer, 1 μℓ of cDNA, 1× PCR buffer and 1 U of Taq DNA polymerase (Invitrogen). The following combinations of forward and reverse primers were used in the reactions: for *CpNPR1*, 5'-TGGCCCTTTATCAGCGGCAC-3' and 5'-TCCTACCTTCAGGGCCTTG-3'; for *CpNPR2*, 5'-TCCATTATGCTGTGGCATAC-3' and 5'-TAACAGAACTCTGCCCATCC-3'; for *CpNPR3*, 5'-TGATTTGGAAGTGGACGCTG-3' and 5'-AGGCCTCGAGGGTTCCTAA-3'; for *CpNPR4*, 5'-ACCTCCCTATTGATGTGGTG-3' and 5'-CTCGACTGCAATTCTCGACA-3'. The position of these primers on the corresponding papaya *NPR1* homologue sequences is depicted in Supplementary Figure 1. Cycling conditions were 95°C for 3 min, followed by 30 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 1 min, followed by a final incubation of 5 min at 72°C. In RT-PCR experiments, the forward 5'-TTCACCCTTGGTGTCAAGC-3' and reverse 5'-TACCAGTCAAGGTTGGTGG-3' primers for the papaya elongation factor 1 alfa (*CpEF1α*) were used as positive or negative control, in this last case to detect any genomic DNA contamination from RNA samples treated with RNase-free DNase and without reverse transcriptase. PCR products were separated by electrophoresis on a 1% agarose gel stained with ethidium bromide and visualized under UV light using an image analysis system GelDoc (BIORAD).

Results and Discussion

The papaya genome contains four *NPR1* homologues

Similarity searches in the papaya genome using the full set of *Arabidopsis NPR1* homologues as queries revealed four *NPR1* homologue genes (Table 1) with the BTB/POZ-ankyrin modular structure predicted in their encoded proteins, including the previous *CpNPR1* sequence reported by Zhu et al. (2003) (GenBank accession no. AAS55117). The other three sequences were named as *CpNPR2*, *CpNPR3* and *CpNPR4* (Fig. S1). These papaya *NPR1* homologue sequences were also used in BLAST searches in the papaya genome, however apart from the four papaya NPR1 sequences already isolated, no other papaya *NPR1* sequence was retrieved. Therefore, the *NPR1* family in papaya is comprised by a small number of members. This finding is consistent with the small number of *NPR1* homologues (3-6) present in other plant genomes (Arabidopsis Genome Initiative, 2000; Goff et al., 2002; Tuskan et al., 2006; Velasco et al., 2007). These data indicate that the BTB/POZ-ankyrin modular structure of the *NPR1* family has not undergone an extensive gene duplication and diversification process in comparison to other plant gene families involved in disease resistance such as NBS-LRR or receptor-like kinase genes (Meyers et al., 2003; Lehti-Shiu et al., 2009). Percent amino acid identity between the predicted amino acid sequences of papaya NPR1 homologues and the *Arabidopsis* NPR1 homologues ranged from 26.1% (CpNPR4 vs AtNPR1) to 87.4% (CpNPR4 vs AtNPR5), whereas amino

acid identity among the papaya NPR1 homologues ranged from 26.8% (CpNPR1 vs CpNPR4) and 56.4% (CpNPR2 vs CpNPR3) (Table 2). A similar percent amino acid identity was found among *Arabidopsis* NPR1 sequences (Table 2).

The CpNPR1 predicted protein contained a N-terminal phosphodegron motif (DSxxxS) (Fig. 1) that is highly conserved in AtNPR1 and AtNPR2 (Spoel et al., 2009). Phosphodegrons are degradation motifs found in many proteasome-regulated substrates, including the mammalian cofactor IkB, which shares structural features with NPR1 and functions

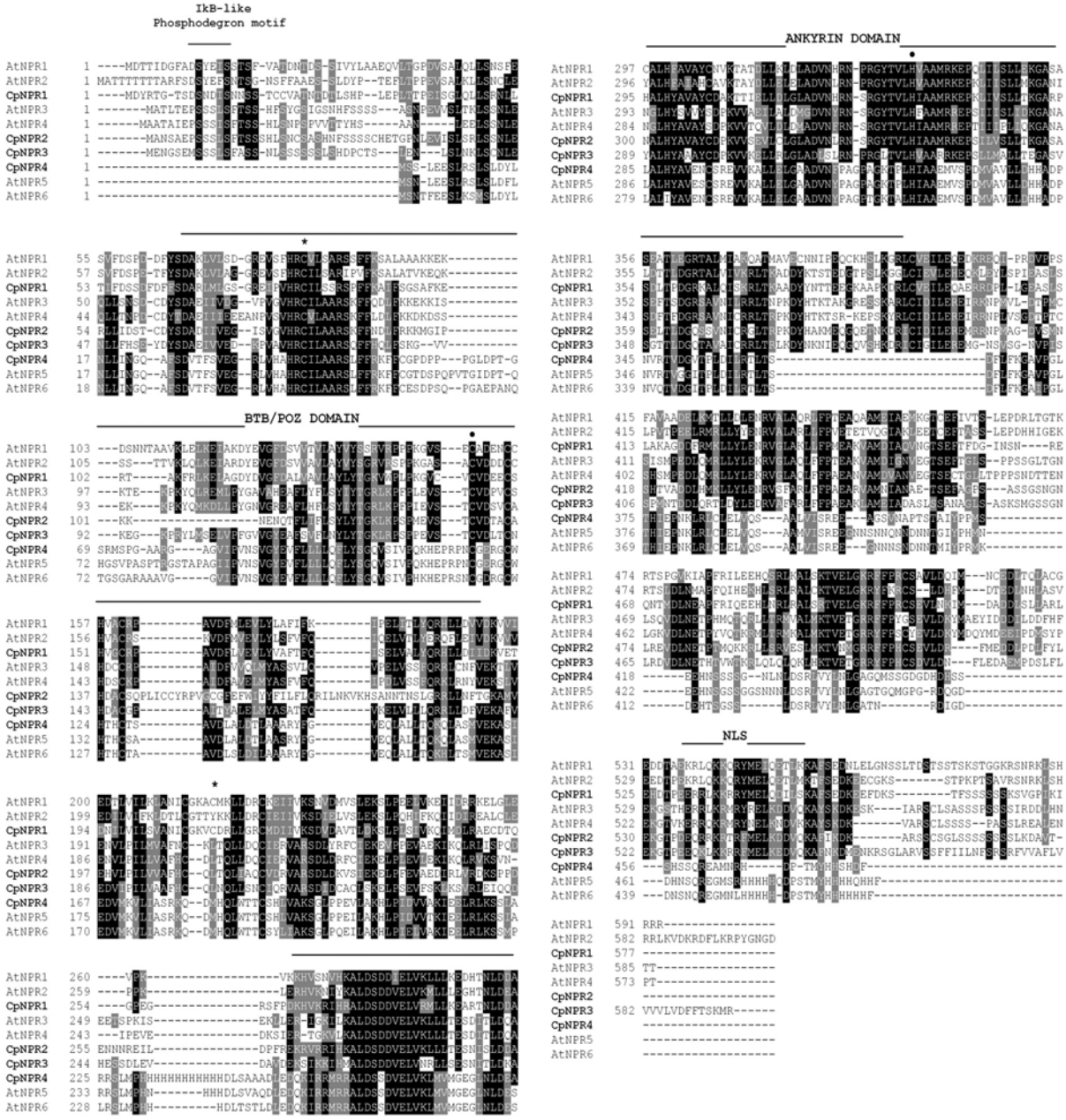

Figure 1. ClustalX alignment of the deduced amino acid sequences of papaya and *Arabidopsis* NPR1 homologues. The BTB/POZ and ankyrin repeat domains are indicated, as well as the IkB-like phosphodegron motif and the nuclear localization signal (NLS) (Cao et al., 1997; Kinkema et al., 2000; Hepworth et al., 2005; Spoel et al., 2009). The mutations *Atnpr1*^{C82A} and *Atnpr1*^{C216A} that lead to constitutive monomerization of the protein, nuclear localization and PR gene expression are indicated with asterisks (Mou et al., 2003). The mutations of *Atnpr1*^{C150Y} and *Atnpr1*^{H334Y} that lead to loss of AtNPR1 function (Cao et al., 1997) and abolish the interaction with AHBP-1b/TGA2 transcription factor (Zhang et al., 1999), respectively, are indicated with black circles. Identical amino acids are shaded in black and conservative substitutions are shaded in grey.

Table 1. BLASTP results of NPR1 homologue sequences found in the papaya cv. SunUp genome sequence using the six *Arabidopsis* NPR1 homologue proteins as queries in the Phytozome database.

Papaya NPR1 homologues	Score	Identity (%)	Similarity(%)	*E* value
CpNPR1[a]	392.9	54.4	71.2	1.3 e-109
CpNPR2	563.5	57.6	72	5.9 e-161
CpNPR3	467.6	52.4	69.4	4.5 e-132
CpNPR4	682.9	80.9	84.9	0

[a]Sequence previously reported by Zhu et al. (2003)

Table 2. Percent amino acid identity between papaya and *Arabidopsis*NPR1homologues.

NPR1 homologues	CpNPR1	CpNPR2	CpNPR3	CpNPR4	AtNPR1	AtNPR2	AtNPR3	AtNPR4	AtNPR5	AtNPR6
CpNPR1		42.7	39.3	26.8	54	53.7	41.6	41.2	26.3	27.4
CpNPR2			56.4	28.7	35.9	35.7	60.8	58.9	27.5	28.1
CpNPR3				30.6	35.5	36.2	51.7	50.9	28.7	29.1
CpNPR4					26.1	26.6	29.4	29.6	87.4	83.4
AtNPR1						63.4	36.1	37.8	25.1	25.8
AtNPR2							38.1	38	25.1	25.5
AtNPR3								73.9	27.9	27.5
AtNPR4									27.9	28.2
AtNPR5										84.9
AtNPR6										

to inactivate the transcription factor NF-kB in the cytoplasm. In response to pathogen attack, IkB is rapidly phosphorylated and targeted for ubiquitin-mediated proteolysis, allowing NF-kB to localize to the nucleus and activate defense genes (Hayden and Ghosh, 2004). In *Arabidopsis*, application of exogenous SA increases phosphorylation of NPR1 Ser11/15 phosphodegron motif in the nucleus facilitating its recruitment to a Cullin3-based ubiquitin ligase for degradation (Spoel et al., 2009). Turnover of phosphorylated NPR1 is required for full induction of PR genes and establishment of SAR (Spoel et al., 2009). By analogy, this phosphodegron motif found in CpNPR1 could be working in a similar way.

Two protein interaction domains were conserved in the papaya NPR1 family. The first was the BTB/POZ domain, which is generally found at the N-terminus of the protein and contains ~115 residues (Zollman et al., 1994). Overall, this domain was conserved in all papaya NPR1 homologues (Fig. 1). The BTB/POZ domain acts as a protein-protein interaction module that is able to both self-associate and interact with non-BTB proteins (Stogios et al., 2005). Functional studies have shown that the BTB domain is involved in transcription repression, cytoskeleton regulation, tetramerization and gating of ion channels and protein ubiquitination/degradation (Stogios et al., 2005). Biochemical and genetic analysis indicate that BTB harboring proteins provide substrate specificity for Cullin-3-based E3 ubiquitin ligases and thus target proteins for degradation via the proteasome (Pintard et al., 2003; Pintard et al., 2004). Interestingly, AtNPR1 is targeted to the proteasome by CUL3-based E3 ligase-mediated ubiquitinylation (Spoel et al., 2009). Although such a role for the BTB domain has not been established, a mutation in this region (*npr1-2*, C150Y) resulted in the loss of AtNPR1 function (Cao et al., 1997). Interestingly, the corresponding C150 is also present in all papaya NPR1 homologues (Fig. 1). The core elements of the BTB fold topology are three conserved β -strands and five α-helices. Many families of BTB/POZ proteins are of the 'long form', with a N-terminal extension of one α-helix and one β-strand. Other proteins have two additional α-helices at the C-terminus (Stogios et al., 2005). We used the CpNPR1 and AtNPR1 sequences as representatives of the NPR1 family to predict their BTB/POZ fold topology using protein structure homology modeling. The crystallographic data of the human speckle-type BTB/POZ domain (PDB Acc. No. 3htm) was used as template and the results are shown in Figure 2. Overall, it was predicted that the papaya and *Arabidopsis* NPR1 proteins also contain the core elements of the BTB fold topology and belong to the class of BTB domains of the long form since one additional α-helix was predicted at the N-terminus and two additional α-helices at the C-terminus (Fig. 2).

The second conserved domain found in all papaya NPR1 homologues was the ankyrin repeat domain, which consists of four repeats of 33 amino acid residues each (Cao et al., 1997). Ankyrin repeats have been observed to exist alone as a single domain protein or in conjunction with other domains in the same protein like in NPR1. Each repeat folds into two antiparallel α-helices followed by a β-hairpin or a long loop (Mosavi et al., 2004). In the case of both CpNPR1 and AtNPR1, the ankyrin helix-turn-helix conformation was also observed in their predicted 3D structures using the crystallo-

graphic data of the human UPLC1 protein (PBD Acc. No. 2b0o) as template (Fig. 2). The ankyrin repeat domain has been identified in a diverse group of proteins involved in cell-cell signaling, cytoskeleton integrity, transcription and cell-cycle regulation, inflammatory response, development, and various transport phenomena (Mosavi et al., 2004). These repeats have been found to be involved solely in protein-protein interactions (Mosavi et al., 2004). Indeed, in the case of AtNPR1 there is evidence showing that the ankyrin domain interacts with the AHBP-1b/TGA2 transcription factor, and substitution of histidine-334 by tyrosine in the ankyrin domain (npr1-1) impairs the interaction with AHBP-1b/TGA2 (Zhang et al., 1999). Similar functional features could occur in the papaya NPR1 family since the corresponding ankyrin domain and H334 are present in all its members (Fig. 1), and we have identified TGA2 homologues in the papaya genome (Idrovo-Espín et al., 2012).

The C-terminal regions of all papaya NPR1 homologues are rich in basic amino acids, a typical characteristic of nuclear localization signals (NLS) (Nigg, 1997). Kinkema et al. (2000) showed through site-directed mutagenesis that five amino residues in the C-terminus of AtNPR1 are essential for its nuclear translocation (Fig. 1). These amino acids are conserved in CpNPR1, CpNPR2 and CpNPR3 suggesting the possibility of being targeted to the nucleus as well.

The papaya *NPR1* homologues were found in four DNA contigs with a total combined length of 106, 274 bp. These DNA contigs also harbored 12 additional predicted ORFs (Table 3). It was interesting to find that *CpNPR1* and *CpNPR2* shared their respective contigs with retrotransposon-like elements (RTLE). This physical proximity of RTLEs with a *NPR1* homologue sequence has recently been observed in sugarbeet (*Beta vulgaris*) where two long terminal repeat (LTR) RTLEs were found about 10 Kb upstream of the sugarbeet *NPR1* sequence. The first RTLE had homology to a Ty1/copia-type retrotransposon and the second had homology

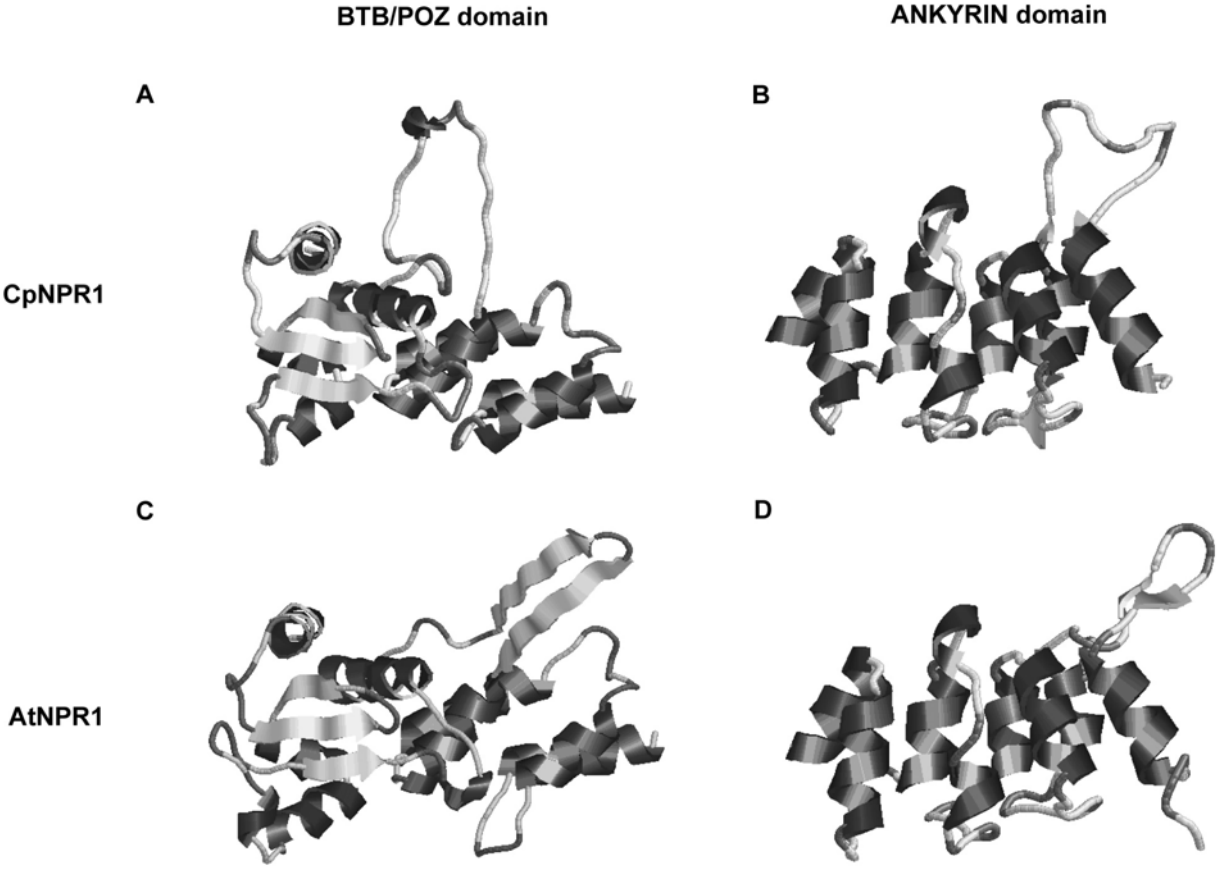

Figure 2. Ribbon view of the predicted three-dimensional structures of papaya and *Arabidopsis* NPR1 proteins. Predicted BTB/POZ and ankyrin 3D domains of papaya (A and B) and *Arabidopsis* (C and D) NPR1 sequences. The crystallographic data of human speckle-type BTB/POZ domain (PDB Acc. No. 3htm) and human UPLC1 ankyrin domain (PDB Acc. No. 2b0o) were used as templates.

to a Ty3/gypsy-type retrotransposon (Kuykendall et al., 2009). There is evidence that in maize the expression of these types of RTLEs are negatively regulated by a RNA-dependent RNA polymerase 2 (RDR2) which is a component of the RNA-directed DNA methylation (RdDM) silencing pathway (Jia et al. 2009). In various reports it has been demonstrated that the expression of transposon elements can decrease the expression of nearby genes (Nigumann et al., 2002; Kashkush et al., 2003; Saze et al., 2007). For example, de-repression of an LTR retrotransposon flanking the *BONSAI* gene in *Arabidopsis* in a chromatin-remodeling factor *ddm1* mutant background causes an epigenetic silencing of *BONSAI* through the production of *BONSAI*-specific short interfering RNAs (Saze et al., 2007). RTLEs are also widely present in disease resistance (R) genes clusters and it has been suggested that they play a role in R gene diversification (Richter and Ronald, 2000) and may also change local methylation patterns (Friedman and Baker, 2007). The location of the RTLEs adjacent to *CpNPR1* and *CpNPR2* is indeed intriguing and encourage further research to determine possible effects of retrotransposons on the expression of *CpNPR1* and *CpNPR2*. *CpNPR2* was also in close proximity to sequences showing homology to a mitochondrial transcription termination factor (mTERF), a membrane-associate protein (TRAM), a pepsin aspartic protease and a small GTP-binding protein (Table 3) which are implicated in different physiological processes such as chloroplast homeostasis (Meskauskiene et al., 2009), lipid metabolism (Winter and Ponting, 2002), disease resistance (van der Hoorn, 2008) and signal transduction (Yuksel and Memon, 2008), respectively. In the case of *CpNPR3*, a RNA helicase homologue was found sharing the same contig (Table 3); plant RNA helicases are involved in RNA metabolism (Umate et al., 2010). It was also interesting to note that *CpNPR4* was located near to genes whose homologues in other plants are involved in development and other physiological processes (Table 3). A homologue to the L-galactono-1,4-lactone dehydrogenase (*GalLDH*) gene was found near to *CpNPR4*. The *GalLDH* gene encodes for an enzyme involved in the synthesis of L-ascorbic acid (AA).

Table 3. Papaya cv. SunUp DNA contigs harboring NPR1 homologues and other genes.

GenBank accession numbers of papaya DNA contigs	Contig length (pb)	Number of predicted ORFs and length (pb)[a]	Homologue to[c]	Accession number	Species	*E* value
ABIM01013147	27947	1 (1731)[b] *CpNPR1*	NPR1-like	ADI24348	*Theobroma cacao*	0
		2 (843)	RVE Retrotransposon	CAN83990	*Vitis vinifera*	5 e-37
		3 (186)	Unknown	-	-	-
ABIM01019889	35066	4 (1983)	Ty3-gypsy Retrotransposon	AAX96626	*Oryza sativa*	1 e-28
		5 (1746) *CpNPR2*	NPR1-like	XP_002520549	*Ricinus communis*	3 e-177
		6 (1737)	mTERF sequence	XP_002332130	*Populus trichocarpa*	0
		7 (822)	TRAM sequence	XP_002263050	*Vitis vinifera*	6 e-130
		8 (1284)	Pepsin aspartic protease	XP_002263357	*Vitis vinifera*	2 e-156
		9 (297)	GTP binding protein	CAA50609	*Nicotiana tabacum*	3 e-10
ABIM01011811	10434	10 (1785) *CpNPR3*	NPR1-like	ACJ45013	*Glycine max*	3 e-172
		11 (2781)	RNA helicase	XP_002307569	*Populus trichocarpa*	0
ABIM01002225	32827	12 (1437) *CpNPR4*	NPR1-like	XP_002531503	*Ricinus communis*	0
		13 (1794)	Lactone dehydrogenase	ACO92659	*Citrus unshiu*	0
		14 (276)	Unknown	-	-	-
		15 (756)	Sec14p	XP_002307993	*Populus trichocarpa*	3 e-113
		16 (579)	Lycopene beta-cyclase	ABD91578	*Carica papaya*	3 e-110

[a] The ORFs encoding for papaya NPR1 homologue proteins are in bold. [b]ORF previously reported by Zhu et al. (2003). [c]The predicted protein sequences were used to perform BLASTP searches in the GenBank. The best BLASTP hit for each ORF is shown.

AA plays a multifunctional role in plants, it directly scavenges reactive oxygen species (ROS), consequently this molecule plays a major role in protecting plant tissues against oxidative damage (Smirnoff, 1996; Smirnoff and Wheeler, 2000). AA is also involved in the process of both cell division and expansion (Smirnoff, 1996; Smirnoff and Wheeler, 2000). A papaya Sec14p homologue gene was also found near to *CpNPR4*. In *Arabidopsis* a homologue of this type of gene was found to be involved in regulating root hair development (Vincent et al., 2005). Another gene in close proximity to *CpNPR4* was a homologue to the lycopene β-cyclase gene (*LCY-B*). The LCY-B enzyme is involved in the synthesis of carotenoid pigments, which are essential for photosynthesis in plants (Pecker et al., 1996). In papaya, there are two lycopene β-cyclase homologue genes, one (*CpLCY-B1*) whose expression is up-regulated in leaves but down-regulated in the flower and fruit (Skelton et al., 2006), and the other one (*CpLCY-B2*) whose expression is up-regulated in the flower and fruit (Devitt et al., 2010). *CpNPR4* was in close proximity to *CpLCY-B1* (Table 3). There are reports of genes located in close proximity and sharing the same biochemical pathway (Salmeron et al., 1996; Watanabe et al., 2000). For example, the *Prf* gene is embedded within a *Pto* kinase gene cluster of ~60 kb. The *Prf* and *Pto* genes encode for a NBS-LRR protein and a kinase protein respectively, and both proteins interact to confer bacterial resistance in tomato (Salmeron et al., 1996). Another example is the pair of *SGL* and *SRK* genes from *Brassica* species, which lie within a distance of ~76 kb (Watanabe et al., 2000). The *SRK* and *SGL* genes encode for a receptor kinase and a secreted glycoprotein, respectively, which are involved in the recognition reaction of self-incompatibility that prevents self-fertilization and promotes outbreeding (Watanabe et al., 2000). By analogy to these examples, the papaya NPR1 homologue proteins could be working in concert with some of the proteins encoded by its neighbor genes to regulate different physiological processes. Further research is required to prove this hypothesis.

The papaya *NPR1* gene family resolves in three phylogenetic clades

The papaya *NPR1* homologues were grouped in three phylogenetic clades, *CpNPR1* in clade I, *CpNPR2* and *CpNPR3* in clade II and *CpNPR4* in clade III (Fig. 3). In each clade there were two representatives of the *Arabidopsis NPR1* family. *CpNPR1* resolved in the same cluster of *AtNPR1* and *AtNPR2* which was supported by a high bootstrap value. This close phylogenetic relationship of *CpNPR1* with *AtNPR1* may suggest a possible function of *CpNPR1* as a positive regulator of SAR in papaya. Likewise, *CpNPR2* and *CpNPR3* may be involved in the negative regulation of SAR as *AtNPR3* and *AtNPR4* do in clade II (Zhang et al., 2006) (Fig. 3). In the case of *CpNPR4*, this gene resolved in clade III along with *AtNPR5* and *AtNPR6* which are involved in leaf and flower development (Hepworth et al., 2005) raising the possibility of a similar function for *CpNPR4* in papaya. The divergence of the papaya *NPR1* homologue genes from the *Arabidopsis NPR1* genes probably occurred ~72 million years ago, which is the estimated date when the *Arabidopsis* and papaya lineages diverged from a common ancestor (Wikström et al., 2001). The most closely related *NPR1* homologue sequences to the four papaya *NPR1* sequences were from *Populus trichocarpa*, *Pyrus pyrifolia* and *A. thaliana*. The finding of *NPR1* homologues from the non-vascular plant *Physcomitrella patens* (clade III) suggests that the modular structure of BTB/POZ-ankyrin is indeed quite ancient since *P. patens* belongs to the Bryophytaea, one of the oldest groups of land plants that originated ~ 700 million years ago (Heckman et al., 2001; Rensing et al., 2008). We did not find *NPR1* homologues in the genome sequence of the microalgae *Chlamydomonas reinhardtii*, *Chlorella variabilis* and *Volvox cartieri*. If we take into consideration that plants and Chlorophytes derived from a common ancestor (Heckman et al., 2001), then we can speculate that the origin of the BTB/POZ-ankyrin modular structure probably coincided with

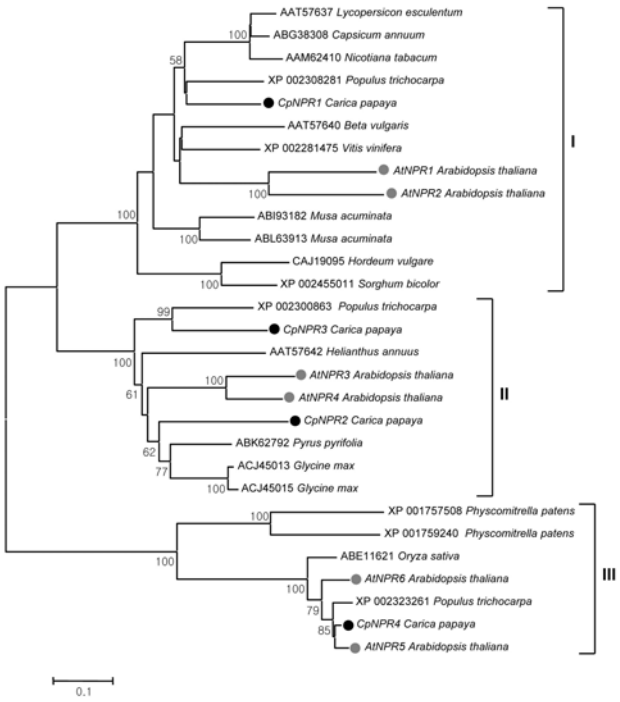

Figure 3. Neighbor-joining phylogenetic tree of NPR1 homologues from vascular and non-vascular plants. NPR1 homologue sequences from papaya and *Arabidopsis* are highlighted with black and grey circles, respectively. GenBank accession number and species name are given for the other NPR1 homologue sequences. Numbers on the branches indicate the percentage of 1000 bootstrap replications supporting the particular nodes and only those with >50% support are shown. Only amino acids spanning from the start of BTB/POZ domain to the end of ankyrin domain were considered for the phylogenetic analysis.

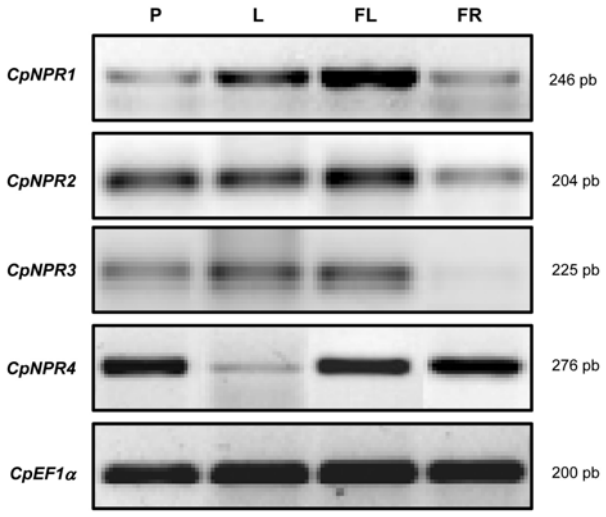

Figure 4. RT-PCR analysis of *NPR1* homologues in *C. papaya* cv. Maradol. Transcript expression of *CpNPR1*, *CpNPR2*, *CpNPR3* and *CpNPR4* was evaluated in different tissues: petioles (P), leaves (L), flowers (FL) and fruits (FR). Primers for the papaya elongation factor (*CpEF1α*) were used as positive control. The numbers on the right are the expected size of the RT-PCR amplicons.

the adaptation of plants to the terrestrial environment.

The papaya *NPR1* homologues are expressed in vegetative and reproductive tissues

The expression of the papaya *NPR1* family was studied in four different tissues (petiole, leaf, flower and fruit) of papaya cv. Maradol using RT-PCR (Fig. 4). Overall, the expression of *CpNPR1*, *CpNPR2*, *CpNPR3* and *CpNPR4* was detected in all four tissues. The expression of *CpNPR1* was previously shown to be present in leaf tissue of papaya cv. Sun Up (Zhu et al., 2003). In the present study, we extended this previous finding by showing that the expression of this gene was also present in leaf tissue of papaya cv. Maradol. The expression of *CpNPR1* in leaf is consistent with previous reports showing the presence of transcripts of this type of gene in leaves of tobacco (Liu et al., 2002), apple (Malnoy et al., 2007), rice (Yuan et al., 2007), grapevine (Le Henanff et al., 2009) and soybean (Sandhu et al., 2009). There are only few reports documenting the basal expression of *NPR1* homologues in tissues other than leaf. Endah et al. (2008) reported the expression of two banana *NPR1* homologue genes in root tissue, while Sandhu et al. (2009) reported the expression of also two soybean *NPR1* homologue genes in stem, flower, young pod and root. In the case of papaya, we provided the first evidence of *CpNPR1* transcripts in petiole, flower and fruit. In the case of *AtNPR3* and *AtNPR4* that are grouped in clade II (Fig. 3), Zhang et al. (2006) showed that these sequences were expressed in leaves of *Arabidopsis*. In papaya, we found that the expression of *CpNPR2* and *CpNPR3* was not only present in leaf but also in the other three tissues examined, although the expression of *CpNPR3* was very low in fruit tissue. The expression of *CpNPR4* was present in all tissues examined which is consistent with the fact that in *A. thaliana,* the expression of *AtNPR5* and *AtNPR6* was present in different types of tissues such as leaves, stems, apex, flowers and roots; but unlike *CpNPR4*, the expression of *AtNPR5* and *AtNPR6* was not present in the silique-type fruit (Hepworth et al., 2005). The basal expression of the papaya *NPR1* family in both vegetative and reproductive tissues suggests that a constant production of transcripts in these tissues is required in order to ensure enough preformed NPR1 proteins ready to respond to upstream signals. No genomic DNA contamination was detected in the RT-PCR reactions and the experiments were repeated twice with similar results (data not shown).

In conclusion, the present study has provided the first comparative analysis of the papaya *NPR1* family members. Each one of the papaya *NPR1* homologues has at least one *Arabidopsis NPR1* counterpart suggesting a common evolutionary origin in terms of structure and possibly in function. Twelve additional ORFs showing homology to retrotransposon elements or genes involved in different physiological processes were found in close proximity to the papaya *NPR1* homologues. Furthermore, the transcriptional analysis of the papaya *NPR1* family members revealed a basal expression in both vegetative and reproductive tissues. These new insights provide a foundation for the functional analysis of the whole papaya *NPR1* family either by overexpressing or silencing through RNA interference. The overexpression of *CpNPR1* may lead to increase resistance to the most damaging pathogens of papaya. On the other hand, the silencing of *CpNPR2* and *CpNPR3* may also lead to enhance disease resistance since their homologues in *Arabidopsis* (*AtNPR3* and *AtNPR4*) work as negative regulators of SAR. Therefore, these gene sequence resources represent new opportunities for engineering disease resistance in this tropical fruit crop.

Acknowledgments This work was funded by the Consejo Nacional de Ciencia y Tecnología (CONACYT, México) project No. CB-83829. Mariana de los Ángeles Menéndez-Cerón and Miguel Ángel Vallejo-Reyna are grateful to CONACYT for the scholarships No. 224295 and No. 228306, respectively. The authors wish to thank José Arjona and Eduardo Castillo for providing plant material and technical assistance, respectively.

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