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ORIGINAL ARTICLE

Apple *NPR1* homologs and their alternative splicing forms may contribute to SA and disease responses

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Abstract The non-expressor of pathogenesis-related genes 1 (*NPR1*) plays essential roles in the salicylic acid (SA) signal pathway and in systemic acquired resistance (SAR) responses. Although a genome-wide analysis of *NPR1* gene family has been conducted in some plant species, little is known about these genes in apple (*Malus* spp.). In this study, eight *NPR1* homologs were identified within the apple genome and 12 different transcripts were cloned by the reverse transcription-polymerase chain reaction. Based on these sequences, the gene structures and sequence alignment of the apple *NPR1* homologs were analyzed. Phylogenetic analysis showed that apple *NPR1* homologs could be classified into three groups as in Arabidopsis. Expression analysis demonstrated that *NPR1* homologs showed different expression patterns in various tissues of apple. Under the induction of SA and MeJA, the transcription levels of some members were upregulated in leaves. Meantime, some *NPR1* genes also showed significantly different expression levels between "Pacific Rose" and *Malus baccata* after inoculation with *Marssonina coronaria*. With the most similarity on amino acid sequence and expression pattern, *MdNPR1* may function as a key regulator in SAR-like *AtNPR1*. These results suggested that the *NPR1* genes may play an important role in plant immune responses, and their alternative splicing may contribute to disease resistance. Our study provides essential information about the *NPR1* homologs in apple and contributes to the understanding of *NPR1* homologs functions in other plants.

Keywords *Malus* · NPR1 · Alternative splicing · SAR · *Marssonina coronaria* · Disease response

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Introduction

Plants are protected from a wide range of pathogens by specialized immune responses, including the hypersensitive resistance (HR), systemic acquired resistance (SAR), and induced systemic resistance (ISR). SAR is an important part of plant defenses, providing long-term, broad-spectrum resistance that is effective against a wide variety of fungal, viral, and bacterial pathogens in tissues distal to the initial site of infection (An and Mou 2011; Friedrich et al. 1996; Robert-Seilaniantz et al. 2011; Sticher et al. 1997). The accumulation of SA or its functional analogs, such as 2,6-dichloroisonicotinic acid, benzothiadiazole S-methyl ester, can activate the SAR response and pathogenesis-related (*PR*) gene expression (Metraux et al. 1991; Ward et al. 1991; Lawton et al. 1995). ISR, normally induced by Jasmonic acid (JA) and ethylene, always confers resistance to insects and some necrotrophic pathogens (Durrant and Dong 2004; Gfeller et al. 2006; Kessler and Baldwin 2002).

The *NPR1* gene was discovered in Arabidopsis mutants with compromised resistance against pathogens (Cao et al. 1994). The *AtNPR1* mutants lost or had reduced transcriptional expression of some *PR*s, including *PR-1* and lost the capacity for SAR, suggesting that the *AtNPR1* gene could prevent secondary infection of virulent pathogens. *NPR1* is also involved in JA/ET-dependent ISR, which is triggered by certain *Pseudomonas fluorescens* strains. *NPR1* has been shown to modulate cross talk between SA- and JA-dependent defense pathways (Dong 2004; Iavicoli et al. 2003; Pieterse and Van Loon 2004). The *AtNPR1* gene encodes a protein that contains two domains, a BTB/BOZ domain and an ankyrin repeat domain (Cao et al. 1997; Ryals et al. 1997). These domains mediate protein-protein interactions and are present in a wide range of proteins with diverse functions (Aravind and Koonin 1999). NPR1 shares structural features with the transcriptional regulator $I_{\kappa}B$, which mediates innate immune responses in animals (Baldwin 1996). A recent review on NPR1 described its mechanisms of action and indicated that NPR1 functions as a bona fide SA receptor (Pajerowska-Mukhtar et al. 2013). During the induction of SAR, Arabidopsis NPR1 protein oligomers in the cytoplasm depolymerize and release active monomeric NPR1, which is translocated into the cell nucleus via a C-terminal bipartite nuclear localization signal (NLS) (Kinkema et al. 2000; Somssich 2003). In the nucleus, NPR1 protein interacts with the TGA (TGACG motif-binding factor) family of basic leucine zipper (bZIP) transcription factors (Despres et al. 2000; Fan and Dong 2002; Kim and Delaney 2002; Subramaniam et al. 2001; Zhang et al. 1999; Zhou et al. 2000). These bZIP transcription factors are involved in SAR and SA-dependent activation of *PR* genes (Niggeweg et al. 2000). In the absence of pathogen challenge, NPR1 is continuously cleared from the nucleus by the proteasome, preventing activation of SAR. SA accumulation promotes NPR1 phosphorylation, which resulted in its recruitment by a Cullin3-based ubiquitin ligase. Turnover of phosphorylated NPR1 is required for full induction of target *PR* genes and the establishment of SAR in plant defense (Mukhtar et al. 2009; Spoel et al. 2009).

The Arabidopsis *NPR1* gene homologs have been extensively studied. Besides *AtNPR1*, five other homologs have been found in Arabidopsis. Phylogenetic analysis of these six homologs revealed three distinct groups (Hepworth et al. 2005; Zhang et al. 2006). *AtNPR1* and *AtNPR2* are in the first group. The function of *AtNPR2* has not been reported, but it is supposed to participate in the SAR and SA signaling pathway as *AtNPR1*. Proteins encoded by the second group, which includes AtNPR3 and AtNPR4, are thought to directly bind to SA with different binding affinity (NPR3, K_d = 981 nM; NPR4, K_d = 46 nM) and play a role in mediating the degradation of NPR1 and decreasing or keeping its function in basal defense (Fu et al. 2012). The members in the third group, encoding Arabidopsis NPR5 and NPR6, also known as BLADE-ON-PETIOLE 1 (BOP1) and BOP2, are associated with the development of lateral organs (Hepworth et al. 2005). A recent report found that the Arabidopsis BOP proteins are responsible for abiotic resistance induced by methyl jasmonate (MeJA) in the wild type (Canet et al. 2012). With the development of genome sequencing, *NPR1* gene homologs have been identified from the whole-genome sequences of many plants, such as *Vitis vinifera* (Le Henanff et al. 2009), *Populus trichocarpa* (Shao et al. 2013), *Carica papaya* (Peraza-Echeverria et al. 2012), and *Persea americana* (Backer et al. 2015). Their functions in disease resistance have been widely reported. Overexpression of the *AtNPR1* gene has been shown to result in enhanced bacterial and fungal resistance in many plants. Furthermore, overexpression of an endogenous *NPR1* orthologue (named *MdNPR3* in our research) in apple increased resistance to fire blight and two other major fungal pathogens (*Venturia inaequalis* and *Gymnospo rangium juniperi-virginianae*) of apple (Malnoy et al. 2007). A study on two banana *NPR1-like* genes, *MNPR1A* and *MNPR1B*, found that they could induce *PR-1* gene expression and restore pathogen resistance as in the *Arabidopsis npr1* mutant (Yocgo et al. 2012). As master regulatory genes in defense responses, the functions of *NPR1* genes have been reported in many studies, but the full complement of gene members and their functions in disease resistance in apple have not been described.

Alternative splicing (AS) is a process that generates two or more different splice variants from the same pre-mRNA molecule using different splice sites. These splice sites are recognized by a large RNA-protein complex, the spliceosome. Four main types of such splicing are known: exon skipping, alternative 5′ and alternative 3′ splice sites, and intron retention. AS not only contributes to proteome diversity but also generates truncated proteins that are potentially regulatory or detrimental to the cell. It also plays a role in gene expression by regulating transcript levels through the production of isoforms that are degraded by the nonsense-mediated decay pathway (Mastrangelo et al. 2012; Syed et al. 2012). In *Citrus clementina*, a defense-related gene, acidic chitinase II, can produce an additional transcript containing a premature termination codon after the first 135 amino acid that is inducible by pathogen infection and MeJA treatment (Del Carratore et al. 2011).

Apple (*Malus domestica* Borkh.) is one of the most important fruit crops cultivated in China and worldwide. Fungal diseases, such as *Marssonina coronaria*, *Glomerella cingulata*, and *Valsa canker*, pose a great threat to the growth and production of apple trees. In this study, eight *NPR1* gene homologs were identified from the apple genome and their full complementary DNA (cDNA) sequences were cloned; furthermore, AS of some *NPR1* homologs were found. The tissue- and organ-specific expression of the *NPR1* homologs and their differential expression in response to *M. coronaria*,

SA, and MeJA induction between resistant and susceptible cultivars were investigated. Some specific members and AS variants of *NPR1* were only found in the resistant cultivar. This implies that these AS forms may be related to disease resistance responses in apple. Our findings provide an invaluable resource for further study and functional characterization of the *NPR1* homologs in apple.

Materials and methods

Identification and annotation of apple *NPR1* homologs

Six Arabidopsis NPR1 homolog protein sequences including AtNPR1–6 were downloaded from The Arabidopsis Information Resource (TAIR, http://www.arabidopsis.org/) with the accession numbers AT1G64280.1, AT4G26120.1, AT5G45110.1, AT4G19660.1, AT2G41370.1, and AT3 G57130.1. Their amino acid sequences were used as queries for BLASTp searches at the Genome Database for Rosaceae (GDR, https://www.rosaceae.org/) with an E-value cutoff of 1e − 003. The results were submitted to the Simple Molecular Architecture Tool (SMART, http://smart.emblheidelberg.de) and Pfam protein family databases (http://pfam.xfam.org/) to detect the presence of BTB/POZ and ankyrin domains. Sequences containing both domains were selected as putative *NPR1* homologs. Afterward, the coding sequences identified from the apple genome data were inspected and revised according to the *M. domestica* and *M. pumila* expressed sequence tags (ESTs), which are available from the NCBI web server (http://www.ncbi.nlm.nih.gov/).

Plant materials and treatments

Five-year-old apple trees of the “Qinguan” and “Pacific Rose” cultivars and a wild apple species *Malus baccata* Borkh were grown in the field under a rain-shelter at the experimental station of Northwest A&F University (Yangling, Shaanxi, China) and used as materials throughout the study. The apple cultivars Qinguan and Pacific Rose were shown to have different levels of resistance to *M. coronaria*, which causes apple brown spot. Pacific Rose was previously reported as susceptible, while Qinguan and *M. baccata* are resistant and highly resistant, respectively, to the disease (Yin et al. 2013).

Tissue- and organ-specific expression of the apple *NPR1* homologs was investigated in Pacific Rose. New shoots (near the apices of newly-growing shoots and 3–4 mm in diameter), leaves (the third to fifth fully expanded young leaves beneath the shoot apices when newly-growing shoots were 40–60 cm in length, without petioles), flowers (with receptacle), pericarp, pulp (from mature fruits of about 7 cm in diameter), and seeds of mature fruit were collected for total RNA isolation and expression analysis.

The induction of *NPR1* expression by SA and MeJA was examined in Qinguan and Pacific Rose, and by *M. coronaria* inoculation in Pacific Rose and *M. baccata*. SA and MeJA treatment was performed by spraying leaves with 100 mM SA or 50 mM MeJA solutions once at 8:00 a.m. on whole sample plants. Leaves sprayed with sterile distilled water were used as controls. Each treatment and control were performed on 15 trees. All treated plants were sampled at 0, 1, 3, 6, 12, and 24 h postinoculation (hpi) for subsequent RNA isolation and *NPR1* homolog expression analysis. *M. coronaria* inoculation was conducted on 90~120 detached leaves according to Li et al. (2014) under a 24-h light/0-h dark photoperiod and 15~20 sample leaves were collected at 6, 24, 48, 72, 96, and 144 hpi. All treatments were performed in triplicate.

RNA extraction and cDNA synthesis

Total RNA was extracted using a modified SDS-LiCl method (Hou et al. 2011). All reagents were prepared with 1 ‰ DEPC treated water. Potassium acetate buffer (pH 5.0) was added to precipitate the SDS and macromolecule material. The chloroform/isoamyl alcohol extraction step was repeated three or four times until the protein phase was completely removed and the water phase became clean. RNA integrity was assessed on 2 % TAE agarose gel under non-denaturing conditions. The first-strand cDNA was synthesized from mature mRNA using the PrimeScriptII RT reagent Kit (Takara, Dalian, China) with Oligo(dT) primers according to the manufacturer’s guidelines. Intron-spanning primers were used to assess genomic DNA contamination.

Molecular cloning of *NPR1* homologs in apple

To confirm the identified apple *NPR1* homolog loci in the apple genome, eight pairs of gene-specific primers targeting the exons were designed based on the putative *NPR1* homolog gene sequences from the apple genome database and the apple EST sequences (Supplementary Table S1). Putative full-length coding sequences of the apple *NPR1* homolog genes were amplified by RT-PCR from both Qinguan and Pacific Rose leaves with cDNA templates instead of genomic DNA because some of the gene fragments were too long to amplify. PCR was performed with *pfu* Taq DNA polymerase (Thermo, Shanghai, China), using the manufacturer recommended reaction conditions. The PCR products were retrieved and purified with a DNA Fragment Purification Kit (Tiangen, Beijing, China). A tails were added to the PCR fragments using r*Taq* (Takara) at 72 °C for 35 min, and the fragments were inserted into the pMD-19T cloning vector (Takara). Positive clones were verified by blue/white colony assays and sequencing. The absence or presence of an AS variant was judged by

agarose gel or sequencing of 10 independent clones from the RT-PCR product of *NPR1s*.

Sequence alignment, phylogenetic analysis, and exon-intron structure analysis of apple *NPR1* homologs

Because the eight deduced apple *NPR1* homolog cDNA sequences and most transcript variants were detected in Pacific Rose, the cDNA sequences of the apple homologs in Pacific Rose were used for subsequent sequence alignment analyses. The apple NPR1 protein sequences deduced from the cDNA fragments were aligned using the program ClustalX with BLOSUM30 as the protein weight matrix. A phylogenetic tree of apple NPR1 protein sequences was constructed with 28 other putative NPR1-like proteins sequences downloaded from the NCBI database from eight plant species (Table 1) except for CpNPR1–4 (the sequences were obtained from the supplementary material of Peraza-Echeverria et al. (2012)). Phylogenetic trees were constructed using the neighbor-joining method by the program MEGA5 (Molecular Evolutionary Genetics Analysis) with the p-distance and complete deletion option parameters selected (Saitou and Nei 1987; Tamura et al. 2011). A diagram of the exon/intron organization was generated by the online Gene Structure Display Server (GSDS, http://gsds.cbi.pku.edu.cn). A gene structure map was generated based on the coding sequence of the apple cultivar Pacific Rose and corresponding genome sequence of apple (Ver 1.0, https://www.rosaceae.org/).

Gene expression detection by qRT-PCR

Real-time quantitative PCR (qRT-PCR) was performed on a Bio-Rad iQ5.0 platform to detect the expression levels of apple *NPR*1 homologs in different tissues and in response to different treatments. The reactions were performed in 20-μL volumes containing 10 μL of SYBR® Premix ExTaq II (Takara), 2.0 μL of cDNA template (50 ng/μL), 1.6 μL of gene-specific primers (10 pmol/L), and 6.4 μL of sterile distilled water. The PCR amplification conditions were 95 °C for 1 min, and then 40 cycles of 95 °C for 10 s, 58 °C for 30 s, and 72 °C for 20 s, followed by melting curve analysis in which the products were heated from 58 to 95 °C by 0.5 °C per cycle. Fluorescence was measured during the extension step of each cycle. Specific primers were designed by primer premier 5.0 for each gene, and each pair was subjected to a local blast search to ensure its specificity (Supplementary

Table 1 Additional NPR1-like proteins from other plants used to construct the phylogenetic tree

Organism	Name	Accession number	Reference
Nicotiana tabacum	NtNPR1	AAM62410.1	
Populus trichocarpa	PtNPR1	XP_002308281.1	(Shao et al. 2013)
	PtNPR2	XP_002322351.2	(Shao et al. 2013)
	PtNPR3	XP_002300863.2	(Shao et al. 2013)
	PtNPR4	XP_002307566.2	(Shao et al. 2013)
	PtNPR5	XP_002323261.1	(Shao et al. 2013)
	PtNPR6	XP_002308905.1	(Shao et al. 2013)
Pyrus pyrifolia	PpNPR1.1	ABK62792.1	
Vitis vinifera	VvNPR1.1	XP_002281475.1	(Gaëlle Le Henanff et al. 2011)
	VvNPR1.2	XP_002274045.1	
Persea americana	PaNPR1	AKH61407.1	(Backer et al. 2015)
	PaNPR2	AKH61408.1	(Backer et al. 2015)
	PaNPR3	AKH61409.1	(Backer et al. 2015)
	PaNPR4	AKH61410.1	(Backer et al. 2015)
	PaNPR5	AKH61411.1	(Backer et al. 2015)
Musa acuminata	MaNPR1.1	ABI93182.1	(Endah et al. 2008)
	MaNPR1.2	ABL63913.1	(Endah et al. 2008)
	MaNPR1.3	ACJ04030.1	
Arabidopsis thaliana	AtNPR1	AT1G64280.1	(Cao et al. 1997)
	AtNPR2	AT4G26120.1	
	AtNPR3	AT5G45110.1	(Liu et al. 2005)
	AtNPR4	AT4G19660.1	(Liu et al. 2005)
	AtNPR5	AT2G41370.1	(Hepworth et al. 2005)
	AtNPR6	AT3G57130.1	(Hepworth et al. 2005)
Carica papaya	CpNPR1		(Peraza-Echeverria et al. 2012)
	CpNPR2		(Peraza-Echeverria et al. 2012)
	CpNPR3		(Peraza-Echeverria et al. 2012)
	CpNPR4		(Peraza-Echeverria et al. 2012)

Table S1). The apple actin gene (GQ339778) was selected as an internal standard (Fan et al. 2011). Three biological replicates were used for each assay. The relative expression level of each gene was calculated according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Results

The published apple genome contains eight *NPR1* gene homologs

A total of eight *NPR1*-like genomic sequences containing both BTB/POZ and ankyrin repeat domains were identified from the apple genome database (Ver 1.0) and named *MdNPR1* to *MdNPR8*. The locations of these apple *NPR1* gene homologs in the apple genome were listed in Table 2. The eight genes were located in seven different chromosomes; two in chromosome 2 and one each in chromosomes 5, 9, 10, 12, 14, and 17. Their location was scattered over different chromosomes and did not show any cluster distribution clues to the expansion of the gene family. Blast analysis using the predicted amino acid sequence of the apple *NPR1* homologs showed that MdNPR1 was most similar to AtNPR1 (49.7 % identity) (Table 3), MdNPR2, MdNPR3, MdNPR4, and MdNPR5 were most similar to AtNPR3 (54.7, 59.9, 59.7, and 53.8 % identity, respectively), and MdNPR6 was most similar to AtNPR4 (51.9 % identity). Furthermore, MdNPR7 and MdNPR8 were most similar to AtNPR5 (77.8 and 77.8 % identity, respectively). No homologs in apple were found to be similar to *AtNPR2* or *AtNPR6*.

Different transcripts of the *NPR1* homologs were found in resistant and susceptible apple cultivars

The full cDNA fragments of all eight *NPR1* gene homologs were cloned and sequenced in the apple cultivars Qinguan and Pacific Rose. A comparison of all the *NPR1* homolog sequences showed that the coding sequences of *MdNPR1*, *3*, *5*, and *6* were the same in both varieties (Supplementary Fig. S1~S8). The *MdNPR4*, *7*, and *8* gene sequences of the two varieties differed in several bases, but the protein sequences were unchanged. The *MdNPR*2 gene sequences of the two cultivars had several different bases leading to two amino acid mutations in the conserved ankyrin domain and two outside the conserved domain region. Two different *MdNPR*2 transcripts with several base differences at different positions were cloned from Qinguan, implying that gene editing may have occurred. A total of 12 transcripts of the eight *NPR1* homologs were found, showing differential splicing of *NPR1* homologs. *MdNPR1*, *2*, *4*, and *5* had two different splicing variants each, while *MdNPR3*, *6*, *7*, and *8* had only one. The variation of spliced size varied from 77 bp which only could be seen by sequencing to 754 bp which could be seen by the agarose gel (Fig. 1). The sequences of the different splicing variants were submitted to GenBank, and their presence or absence in the resistant and susceptible apple cultivars is shown in Table 2. According to the sequences (Supplementary Fig. S1~S8), different forms of alternative splicing were found for the *NPR1* homologs (Fig. 2). An alternative 3′ splicing site in the last exon was found in *MdNPR1*, an alternative 5′ splicing site in the second exon and an alternative 3′ splicing site in the last exon was found in *MdNPR*2, intron retention in the third intron was found in

Table 2 *NPR1* homologs' distribution in genome and their alternative splicing variants in apple cultivars

Gene ID	Matched ID	Genomic position	Coding region length	Score	Variants name	Accession number	Amplified cDNA length	Protein size (aa)	Variants in 'Qinguan'	Variants in 'Pacific Rose'
NPR1	MDP0000292425	chr2:6,260,969.6267058	2822	494 e-139	*MdNPR1a*	KU166911	1835	590	−	+
					MdNPR1b	KU166912	1758	556	+	+
NPR2	MDP0000300400	chr5:8,290,799.8301535	2691	310 4e-84	*MdNPR2a*	KU166913	1796	580	+	+
					MdNPR2b	KU166914	1215	393	+	+
NPR3	MDP0000868782	chr10:24,387,288.24389546	2259	336 5e-92	*MdNPR3*	KU166915	1891	586	+	+
NPR4	MDP0000484484	chr2:1,409,008.1411304	2297	322 6e-88	*MdNPR4a*	KU166916	1879	587	+	+
					MdNPR4b	KU166917	1970	503	−	+
NPR5	MDP0000241323	chr9:11,163,451.11170694	3409	310 4e-84	*MdNPR5a*	KU166921	1820	590	+	+
					MdNPR5b	KU166920	1066	228	+	+
NPR6	MDP0000177622	chr17:11,430,257.11436444	2625	276 5e-74	*MdNPR6*	KU166922	1886	585	+	+
NPR7	MDP0000210506	chr14:13,175,961.13178404	2444	113 7e-25	*MdNPR7*	KU166918	1688	498	+	+
NPR8	MDP0000167136	chr12:10,382,776.10385321	2546	112 1e-24	*MdNPR8*	KU166919	1659	493	+	+

"+" indicates the variant is present in this cultivar. "−" means the variant is absent from this cultivar

Table 3 Identity of putative amino acid sequences of *NPR1* homologs between apple and Arabidopsis

NPR1 homologs	*MdNPR2*	*MdNPR3*	*MdNPR4*	*MdNPR5*	*MdNPR6*	*MdNPR7*	*MdNPR8*	*AtNPR1*	*AtNPR2*	*AtNPR3*	*AtNPR4*	*AtNPR5*	*AtNPR6*
MdNPR1	39.8	42.9	42.7	40.4	39.6	30.3	29.4	**50***	50	40	41	31	32
MdNPR2		73.7	73.3	57.1	56.4	33.3	32.3	35	37	**55**	53	31	30
MdNPR3			**90.6**	60.2	58.7	32.3	32.5	38	39	**60**	58	31	32
MdNPR4				60.5	60	31.9	29.8	38	37	**60**	60	31	33
MdNPR5					**85**	34.3	33.5	35	36	**54**	53	34	33
MdNPR6						32.9	31.9	35	36	51	**52**	34	31
MdNPR7							**94**	26	28	28	30	**78**	73
MdNPR8								26	28	29	30	**78**	74
AtNPR1									61	38	39	28	29
AtNPR2										37	38	29	28
AtNPR3											69	30	30
AtNPR4												31	30
AtNPR5													79

* The bold figure represent each apple NPR1 member most homology to NPR1 member of apple or Arabdopsis

MdNPR4, and exon skipping in the second exon was found in *MdNPR5*. The splicing variants *MdNPR1a* and *MdNPR4b* were only found in Pacific Rose, but other splicing variants were found in both cultivars.

Conserved domain and cysteine site mutations were found in some NPR1 homologs

Multiple sequence alignment was carried out to examine the conservation of the domains in the apple NPR1 proteins at each residue position. The results showed that the apple NPR1 proteins all contain the BTB/POZ and ankyrin repeat domains, except MdNPR5b, which had lost the ankyrin repeat domain (Fig. 3). The 12 apple NPR1 homolog proteins all harbored conserved cysteine residues at C82, C150, C155, and C160, but only the MdNPR1a and MdNPR1b proteins had a conserved cysteine at C216. A key cysteine at C521/529 in AtNPR1 was not conserved in any of the apple NPR1 proteins. These cysteine sites are thought to be important for the formation of functional oligomers, and the mutation or deletion of these cysteine sites may result in functional changes of the NPR1 proteins. The normal transcripts of the *MdNPR1-MdNPR6* contained a NPR1_like_C region and NLS in the C-terminus of their protein. However, the alternative splicing variants *MdNPR1b*, *MdNPR2b*, *MdNP4b*, *MdNPR5b*, *MdNPR7*, and *MdNPR8* did not have the NLS in their C-terminus; their function and localization need further investigation.

Gene structure and phylogenetic analysis of the apple *NPR1* homologs

Gene structure analysis of the apple *NPR1* homolog sequences indicated that they had similar exon-intron structures to the

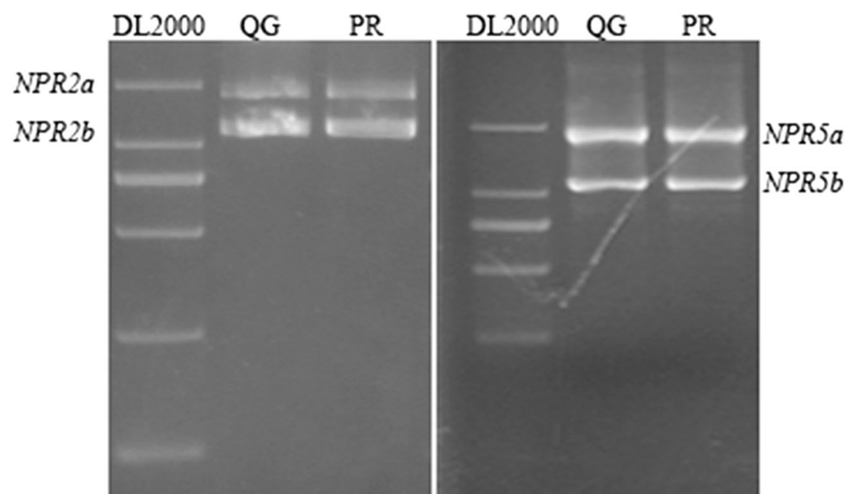

Fig. 1 The RT-PCR result of *NPR2 and NPR5* gene homologs from apple. QG and PR indicate the lane from 'Qinguan' and 'Pacific Rose' respectively; DL2000 is a DNA lander including 2000, 1000, 750, 500, 250, and 100-bp stripes; **a**, **b** indicates different variants from alternative splicing

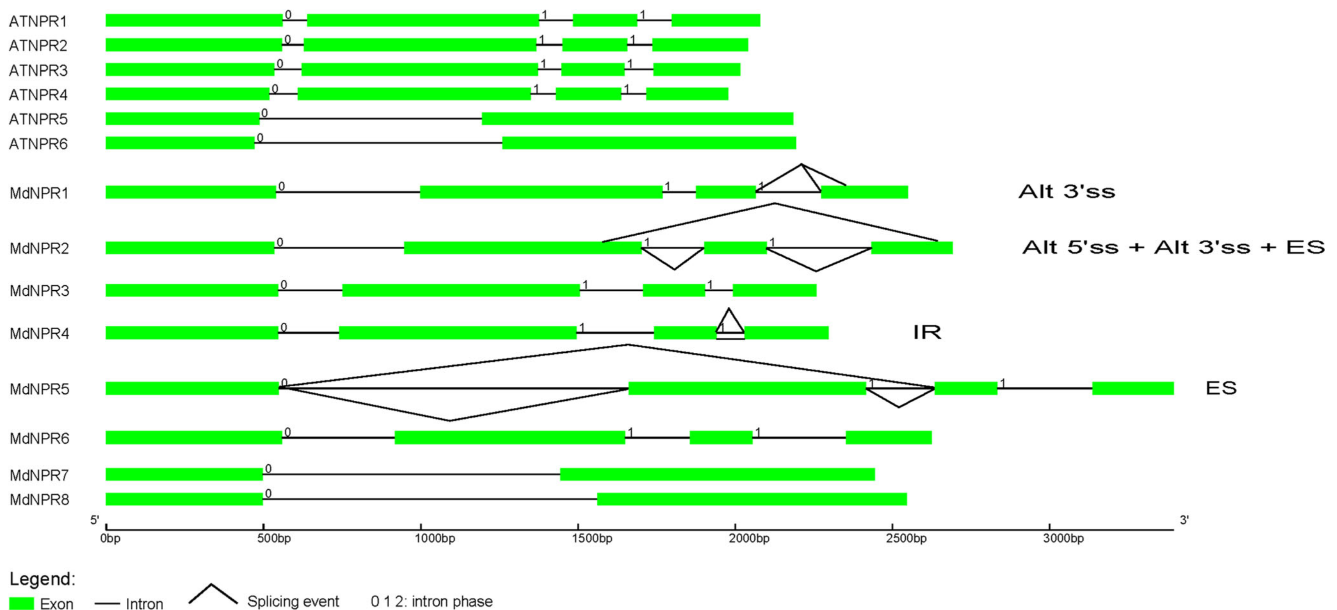

Fig. 2 Exon-intron structures of *NPR1* gene homologs from apple and *Arabidopsis*. *Alt 3'ss* alternative 3′ splice sites, *Alt 5'ss* alternative 5′ splice sites, *ES* exon skipping, *IR* intron retention

NPR1 genes of Arabidopsis. The homologs in group I and II had four exons, while those in group III had two. In addition, the lengths of corresponding exons in each group were almost the same (Fig. 2).

To study the evolutionary relationships among the *NPR1* homologs, a phylogenetic tree was constructed with 28 putative NPR1-like proteins from eight plant species (Fig. 4). According to the results, all the selected *NPR1* homologs were clustered into three groups, which was consistent with previous findings in Arabidopsis. In group I, only MdNPR1 was located in the same cluster as AtNPR1 and AtNPR2. Notably, Group II had the most numbers of apple *NPR1* homologs (five homologs) compared with only two from *Arabidopsis*, two from *Carica papaya*, three from *Populus trichocarpa*, and one from *Persea Americana*. *MdNPR2*, *MdNPR3*, and *MdNPR4* were closely clustered with *PpNPR1.1*, *AtNPR3*, and *AtNPR4*. *MdNPR5* and *MdNPR6* were clustered closely with *CpNPR3*, *PtNPR3*, and *PtNPR4*. Two members (*MdNPR7* and *MdNPR8*) were classified into group III, which was called the BOP group in reference to Arabidopsis.

Differential expression levels of *MdNPR1* homologs in apple tissue and organs

Investigating the specific expression of a gene in different tissues and organs can help infer its major function. The expression of all eight apple NPR1 homologs was examined in stems, flowers, leaves, pericarp, pulp, and seeds of Pacific Rose (Fig. 5). The results showed that their expression levels in different tissues were significantly different. The expression level of *MdNPR1* was highest in pulp (2.09-fold), followed by leaves and relatively low in other tissues. *MdNPR2*, *MdNPR3*, and *MdNPR*4 were expressed at a relatively high level in pericarp compared with leaves, stems, pulp, and seeds. The expression levels of *MdNPR2* and *MdNPR*3 were lowest in flowers, while the expression of *MdNPR4* was highest in flowers. *MdNPR5* was mainly expressed in pericarp and pulp. *MdNPR6* expression was also highest in pulp (18.52-fold) like the *MdNPR1* but was moderate in pericarp and seeds and low in leaves, flowers, and stems. Interestingly, the transcription levels of both *MdNPR*7 and *MdNPR*8 were relatively high in flowers and relatively low in stems, leaves, and seeds, but the expression of *MdNPR*7 in fruits was high, while the expression of *MdNPR8* in fruits was very low.

The expression of apple *NPR1* homolog responses to SA and MeJA inoculation

Since *NPR1* homologs are thought to play important roles in plant defense responses, the expression of the apple genes under SA and MeJA inoculation was compared between the resistant cultivar Qinguan and susceptible cultivar Pacific Rose (Fig. 6).

Following SA induction, *MdNPR2*, *MdNPR3*, *MdNPR5*, and *MdNPR6* were downregulated at 1 hpi in the resistant cultivar Qinguan, and then *MdNPR2~7* expression was significantly upregulated at 6 hpi, while *MdNPR8* was upregulated later at 12 hpi compared with controls. Similarly, in SA-induced susceptible Pacific Rose, the expression of *MdNPR*2–*MdNPR*8 was first downregulated at 1 hpi, and then some of the genes were upregulated and reached a peak (*MdNPR2* at 6 hpi; *MdNPR4* and *MdNPR5* at 3 hpi; *MdNPR3* and *MdNPR6* at 12 hpi). Considering the average expression levels between the cultivars, the apple *NPR1* gene

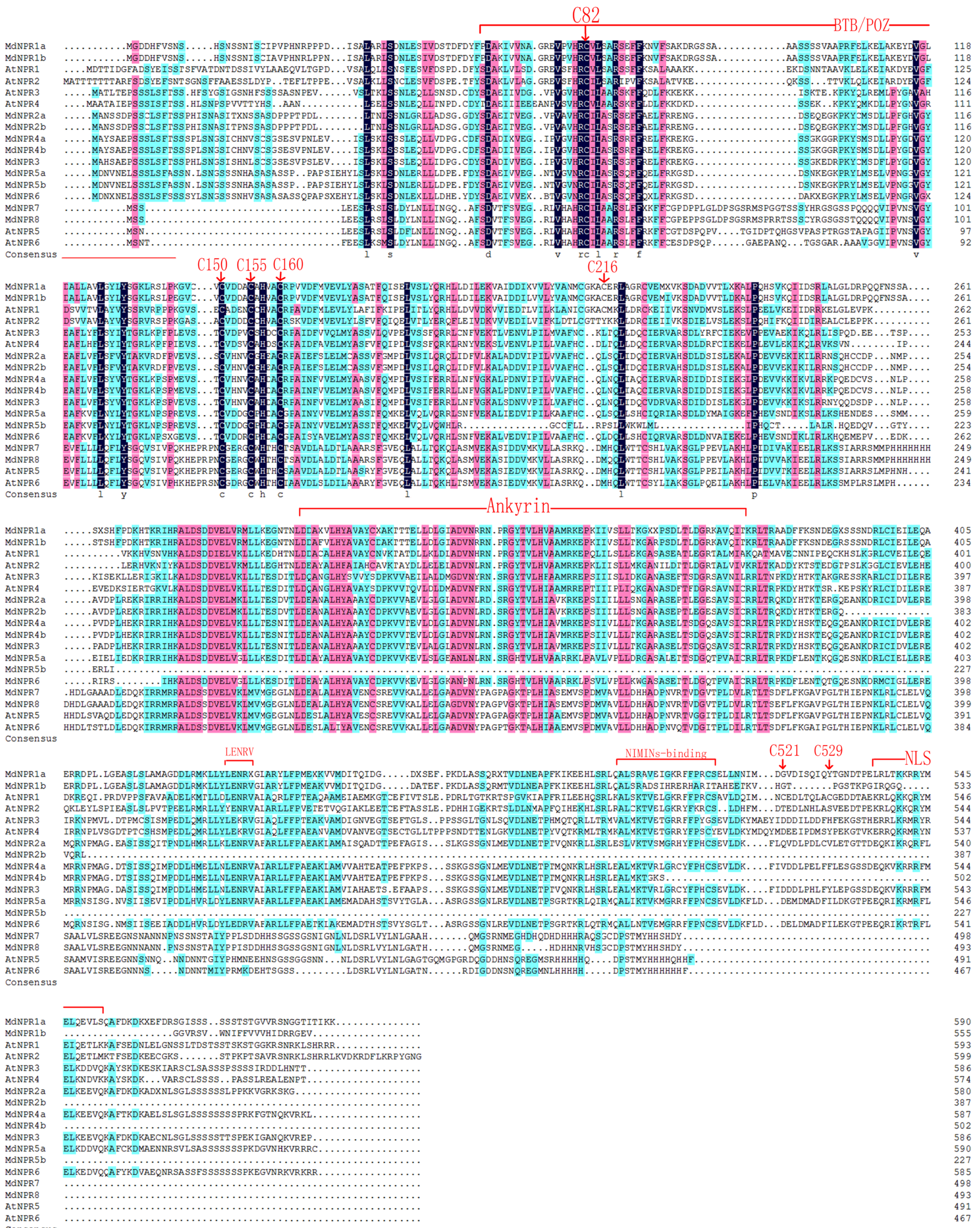

Fig. 3 Multiple alignment of amino acid sequences of *M. domestica* and *Arabidopsis* NPR1 homologs

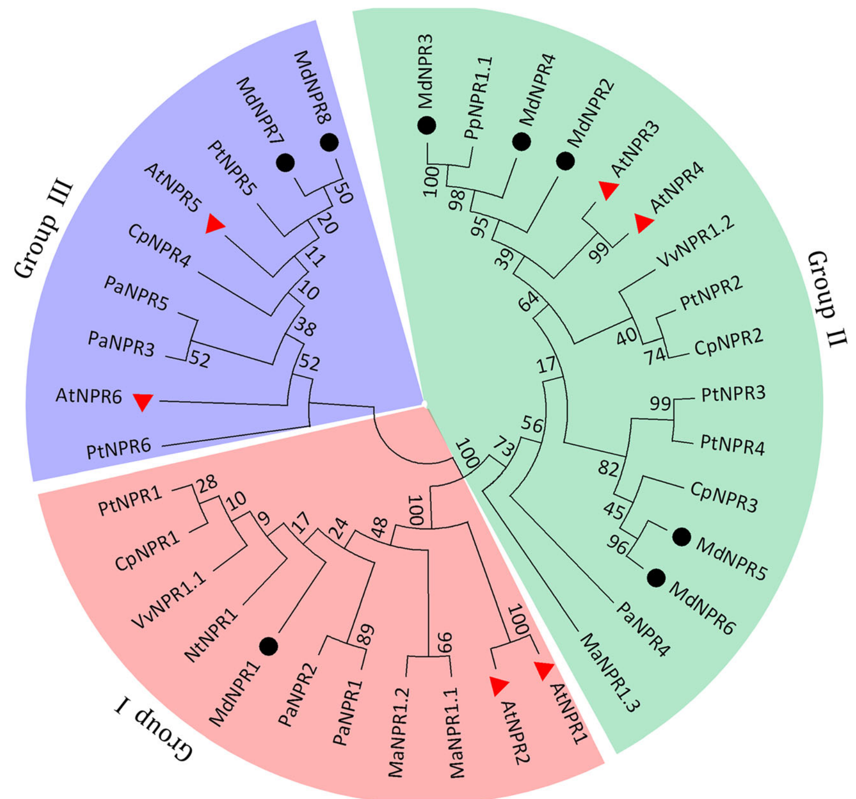

Fig. 4 Phylogenetic analysis of the NPR1 homolog proteins from *M. domestica* and other plant species. The tree was generated with the MEGA software Ver. 5.0 using the neighbor-joining method. A total of 1000 bootstrap replicates were performed, and values are indicated above the branch points

homologs were more highly expressed in Qinguan than Pacific Rose, except for *MdNPR5* at its peak point. However, the *MdNPR4* and *MdNPR5* genes responded earlier in Pacific Rose than in Qinguan.

Following MeJA induction, the expression level of *MdNPR2* was significantly upregulated at 1 hpi in Pacific Rose and at 3 hpi in Qinguan. The expression of *MdNPR3* in Qinguan was first downregulated at 1 hpi and then upregulated. *MdNPR3* was upregulated at 1 hpi in Pacific Rose. *MdNPR*4 expression was sharply upregulated in Pacific Rose at 3 hpi but only slightly upregulated at 12 hpi in Qingquan. *MdNPR5* was upregulated at 0–1 hpi in both varieties and then immediately downregulated until 12 hpi in Pacific Rose, while the expression began to drop at 6 hpi in Qinguan. The expression of both MdNPR6 and MdNPR7 was upregulated at 1 hpi in Pacific Rose, while only MdNPR7 was upregulated at 6 hpi in Qinguan. *MdNPR8* expression was significantly downregulated at 3 hpi in Pacific Rose but upregulated in Qinguan at 12 hpi. The expression of the *MdNPR1* gene was sharply upregulated at 6 and 12 hpi with SA and MeJA, respectively, in Qinguan, but no change was observed in Pacific Rose.

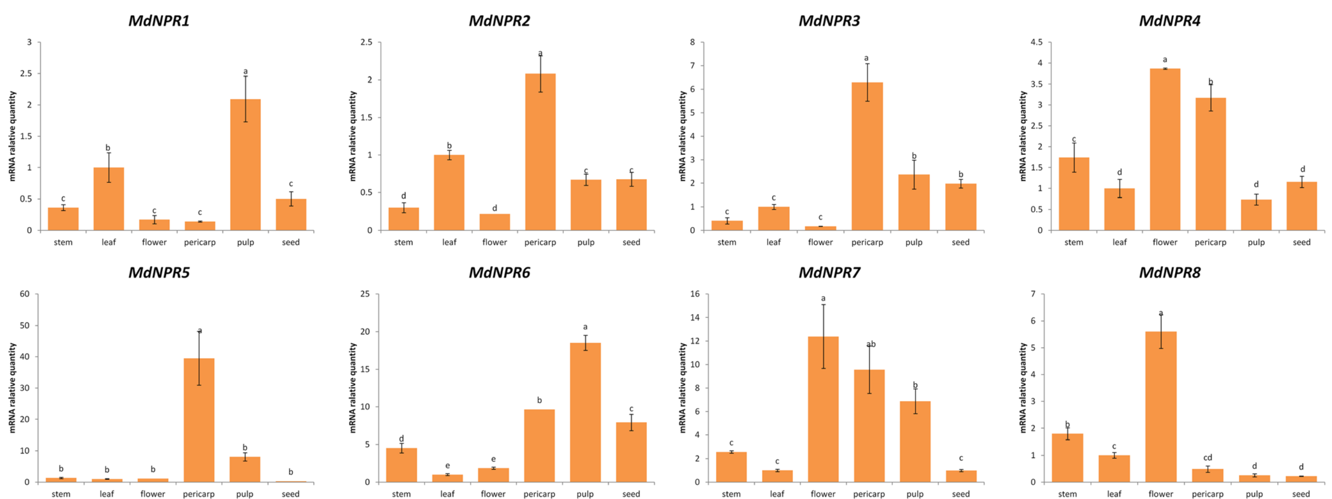

Fig. 5 Expression of apple *NPR1* homologs in various tissues of 'Pacific Rose'

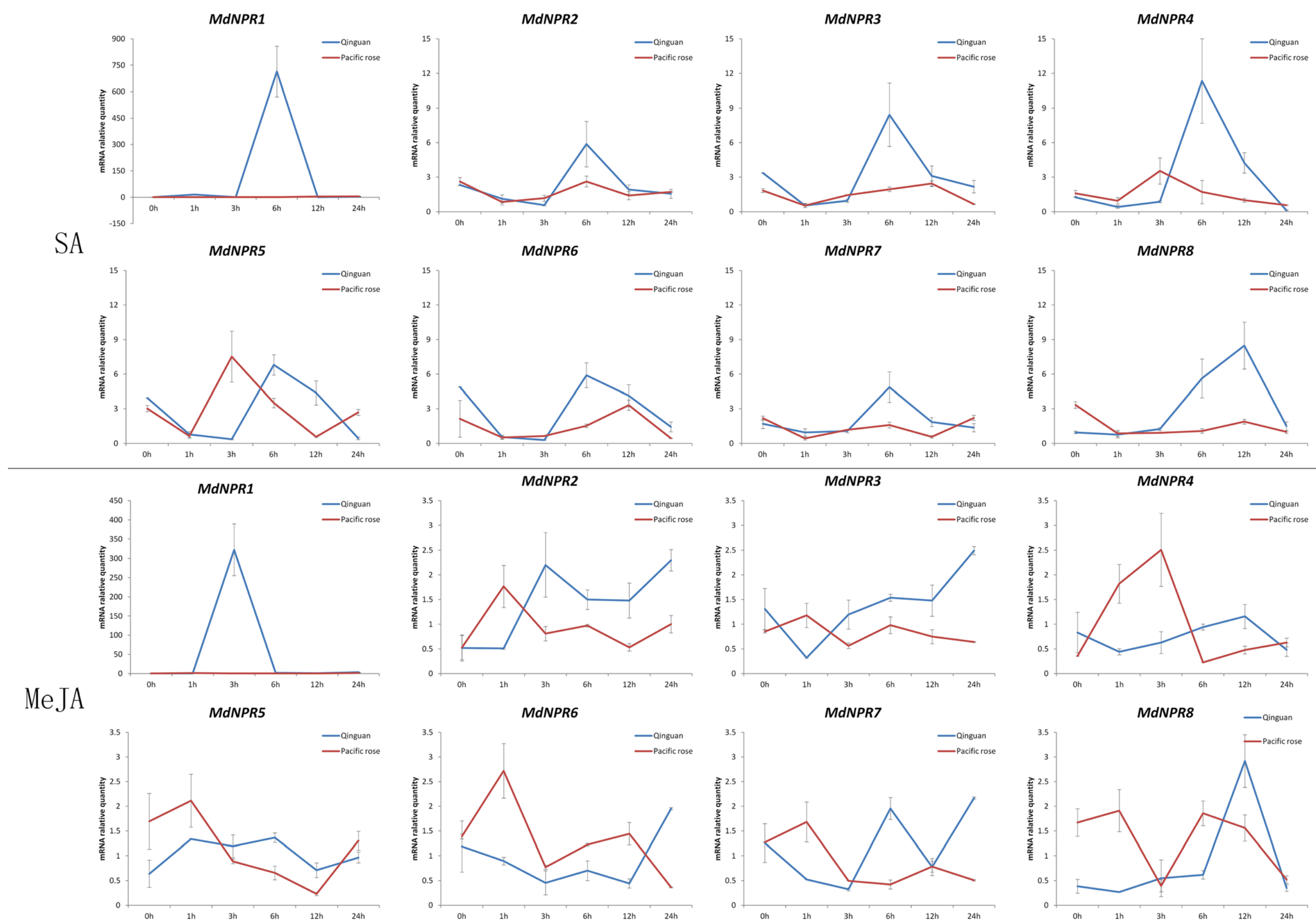

Fig. 6 Expression of apple *NPR1* homologs in response to SA and MeJA in 'Qinguan' and 'Pacific Rose'

Apple *NPR1* gene expression in response to *M. coronaria* in the susceptible apple cultivar and the resistant wild species

The expression changes of the apple *NPR1* homologs were compared between the susceptible apple cultivar Pacific Rose and resistant wild species *M. baccata* after challenge with *M. coronaria* (Fig. 7). The expression of *MdNPR1* was downregulated in *M. baccata* but upregulated and reached a peak in Pacific Rose at 48 hpi. There was also a big decrease in the expression of *MdNPR5* in *M. baccata* at 24 hpi. The *MdNPR*2 gene was upregulated at 48 hpi in Pacific Rose but showed no change in *M. baccata*. Conversely, the expression of *MdNPR6* was upregulated at 48 hpi in *M. baccata* but showed no change in Pacific Rose. The expression of *MdNPR3* in the two varieties was significantly different at 6 hpi but almost the same at all other timepoints. *MdNPR4* expression was significantly higher in Pacific Rose at 96 hpi. The expression of *MdNPR7* and *MdNPR8* showed the same expression trends in both varieties. Interestingly, *MdNPR7* was upregulated at 24 hpi, while *MdNPR8* were downregulated at the same timepoint; subsequently, *MdNPR7* expression returned to a normal level, but the expression of *MdNPR8* was upregulated.

Discussion

Identification of *NPR1* homologs in apple

A large number of studies have revealed that *NPR1* homologs play key roles in various biotic or abiotic responses. However, although *NPR1* genes have been isolated and characterized in several plant species, there is little information about *NPR1* homologs in apple. In this study, eight novel *NPR1* homologs were identified in the apple genome. The *NPR1* homolog in apple is the largest community compared with estimates for other plant species (six members in *Arabidopsis*, four in *Carica papaya* (Peraza-Echeverria et al. 2012), six in *Populus trichocarpa* (Shao et al. 2013), and five in *Persea Americana* (Backer et al. 2015)). The encoded protein sequences of all eight gene homologs included the BTB/POZ and ankyrin repeat domains; these two domains enable the apple *NPR*1 homologs to perform their functions, possibly by interacting with other proteins, like TGA and NIMIN1/2. The NPR1-like-C domain was found in the group I and II members, and is also thought to be an essential component of NPR1 proteins of Arabidopsis

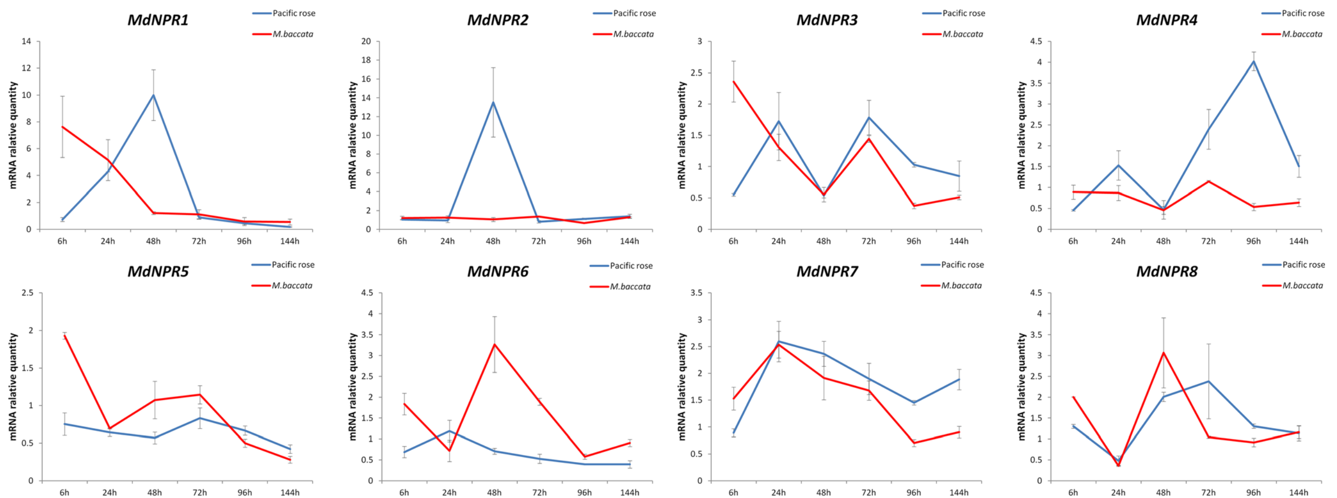

Fig. 7 Expression of apple *NPR1* homologs in 'Pacific Rose' and *M. baccata* after inoculation with *M. coronaria*

(Cao et al. 1997). The NLS in the C-terminus of the NPR1 homologs is essential for nucleus translocation (Kinkema et al. 2000; Maier et al. 2011). C-terminal deletion may have led to localization and functional changes in MdNPR1b, MdNPR2b, MdNPR4b, MdNPR5b, MdNPR7, and MdNPR8. The MdNPR1 and AtNPR1 proteins harbor the conserved cysteine residues Cys82 and Cys216 in the same position, indicating that the MdNPR1 protein may form an oligomer in apple-like AtNPR1. Additionally, Cys150, Cys155, or Cys160 is also highly conserved in all the apple *NPR1* homologs. Mutations in these residues reduced the accumulation of NPR1 (Durrant and Dong 2004). A recent study reported that AtNPR1 could bind SA through the copper ion associated with Cys521/529 (Wu et al. 2012). However, no other NPR1 homolog has cysteine residues in the same site. Thus, the apple *NPR1* may bind to SA with other domains, such as the novel NPR1 domain LENRV or NIMINs-binding sites (Canet et al. 2010; Pajerowska-Mukhtar et al. 2013).

Sequence alignment showed that putative proteins of *MdNPR3* and *MdNPR4* (90.6 % identity), *MdNPR7* and *MdNPR8* (94.4 % identity), and *MdNPR5* and *MdNPR6* (85 % identity) are highly similar in amino acid sequence (Table 3). These data indicate that the *NPR1* homologs in apple may have undergone an extensive gene duplication and diversification process in comparison with other plants.

Interestingly, our phylogenetic analyses indicated that the numbers of homolog members in each of the three subgroups were significantly different. The *MdNPR1* gene in the first group which is highly homologous to *AtNPR1* has never been reported in apple, which means it may function as a key regulator in SAR as *AtNPR1*. The NPR1-like gene, which has been studied before in apple (Malnoy et al. 2007), named *MdNPR3* is classified into the second group. This group also included another four apple *NPR1* homologs, suggesting that their roles and interactions in plant immune responses are complicated. The two members in the third group, *MdNPR7* and *MdNPR8*, share 94.4 % identity in protein sequence, which is higher than those in Arabidopsis (78.6 % identity), indicating that *MdNPR7* and *MdNPR8* are possibly newly evolved in apple.

Molecular cloning of the apple *NPR1* cDNA homologs sequences showed the common existence of AS of *NPR1*, which has never been found in other species before. This AS made the transcription regulation patterns of *NPR1* complicated in apple. The AS in *MdNPR1* and *MdNPR4* did not remove the two conserved domains but removed the NLS at the C-terminus, their effect on the functions need further study. In particular, MdNPR1b was found in both Qinguan and Pacific Rose. MdNPR1a and MdNPR1b may form oligomers individually or together in the cytoplasm. In SAR-induced cells, more MdNPR1a may be transcribed so that more functional NPR1 can be transported to the nucleus. In non-induced cells, more MdNPR1b may be transcribed to form oligomers with MdNPR1a in the cytoplasm. Thus, AS of the MdNPR1 transcript may make nuclear transport of NPR1 proteins faster and more convenient. In addition, *MdNPR5* can lose its second exon during transcription, resulting in a small protein containing only the BTB/POZ domain. The transcription variant of the *MdNPR2* gene can also be translated to a shorter protein but still harbors the two conserved domains. The different sequences of *MdNPR2* in the two varieties indicate alleles or RNA editing of this gene. These proteins may lose their original functions or play other roles in plants. Because we could not isolate *MdNPR1a* or *MdNPR4b* from Qinguan, it is possible that some of the transcriptional variants are specifically expressed in different apple varieties. These transcriptional variants may respond to specific signals or pathogens. Alternative splicing may be an important form of regulation for these genes.

Expression of the apple *NPR1* homologs

The Pacific Rose and Qinguan apple cultivars are generally considered to have different resistance to many diseases. The expression of the apple *NPR1* homologs showed significant differences after induction with SA and MeJA. The apple *NPR1* homologs in group II were more highly expressed in Qinguan than in Pacific Rose after SA induction. In banana cultivars that are resistant to Fusarium oxysporum, *NPR1* (which is homologous to *MdNPR1*) is upregulated earlier and to a greater extent after SA treatment than in susceptible cultivars (Endah et al. 2008). The *MdNPR3* gene was upregulated at 6 h after SA treatment in Qinguan. The result is the same as the previous findings that *MhNPR1* (which is homologous to *MdNPR3*) and the SAR marker genes *MhPR1* and *MhPR5* can be induced by SA (Zhang et al. 2012). However, no significant expression change of *MdNPR3* was found in the susceptible Pacific Rose after SA induction. Given that there was no difference in *MdNPR3* sequence between the two varieties, the transcript levels of this gene may be regulated in different ways. The *MdNPR*4 and *MdNPR*5 genes responded earlier in susceptible Pacific Rose than in resistant Qinguan after SA treatment, and we speculate that these two genes may play a more important role in the susceptible cultivar Pacific Rose.

The specific expression of the *NPR1* homologs was studied in six different apple tissues. The transcription level of the *MdNPR1* gene was very low in all tissues, while relatively high in leaves and fruit, which were consistent with previous findings. *MdNPR2*, *MdNPR3*, *MdNPR5*, and *MdNPR6* were highly expressed in the pericarp and pulp, with the expression of *MdNPR6* in pulp being the highest. Thus, it seems that these four *NPR1* homologs are more important in fruit. *MdNPR4*, *MdNPR7*, and *MdNPR8* expression was highest in flowers, which may mean that these genes play important roles in flower development.

Conidia of *M. coronaria* are widely thought to germinate on the apple leaf surface at about 6 hpi and symptoms (disease spot) can be visually observed at 6 days after inoculation in Pacific Rose (Dang et al. 2011). *MdNPR1*, *MdNPR3*, *MdNPR5*, and *MdNPR6* were highly expressed in *M. baccata* compared with Pacific Rose at 6 hpi. The expression of *MdNPR2* and *MdNPR4* genes fluctuated around only a little after inoculation in *M. baccata*. Interestingly, significant expression differences of *MdNPR1*, *MdNPR2*, and *MdNPR6* were observed between Pacific Rose and *M. baccata* at 48 hpi. Thus, the different resistance of apple to *M. coronaria* could be explained in two ways. Firstly, one of genes may be the key gene affecting resistance, such as the *MdNPR2* gene, which showed polymorphism in Qinguan and Pacific Rose. Secondly, the different transcription levels of the transcription variants after inoculation and their interaction may be a predominant factor in apple. The expression of *MdNPR*7 and *MdNPR8* showed the same change trend after inoculation in both cultivars, suggesting that these two genes do not contribute to the differences in *M. coronaria* resistance in apple.

The functions of the *NPR1* homologs are more complicated in apple than in Arabidopsis. At the protein level, it was speculated that two or more homologs may combine with each other or with signal molecules to regulate the balance of the MdNPR1. NPR1 homeostasis is controlled by SA binding to NPR3/NPR4 in a concentration-dependent manner, which have been proved in Arabidopsis (Moreau et al. 2012). At the transcription level, the apple *NPR1* homolog genes can be regulated by transcription factors or transcribed to different transcripts. This complicated phenomenon should help to maintain the stability of the plant immune system.

In conclusion, this study provides evidence to help the preliminary *NPR1* gene information and functional annotation of the eight newly discovered *NPR1* homologs from apple. Sequence structure and homology as well as phylogenetic analysis suggest that six apple *NPR1* homologs (*MdNPR1–6*) may be involved in defense responses, while the remaining two (*MdNPR7* and *8*) are most likely involved in tissue development. In particular, the expression level of *MdNPR1* was very low but changed rapidly after SA and pathogen treatments. In addition to the most similarity to *AtNPR1* on amino acid sequence, *MdNPR1* gene may function as a key regulator of SAR. The hormone and pathogen inoculation treatment-induced expression, as well as specific expression in various tissues, of the apple NPR1 homologs support this and provide a foundation for future research. Future efforts will be focused on the intracellular interactions and localization of the NPR1 proteins. Additionally, the expression levels and functions of the different transcript variants of *NPR1* homologs in apple need to be explored in depth.

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Data archiving statement All the apple *NPR1* gene homologs cDNA sequence cloned from apple cultivar ‘Pacific Rose’ were submitted to GenBank. Their gene ID and GenBank accession numbers were list as follows: *MdNPR1a* (KU166911), *MdNPR1b* (KU166912), *MdNPR2a* (KU166913), *MdNPR2b* (KU166914), *MdNPR3* (KU166915), *MdNPR4a* (KU166916), *MdNPR4b* (KU166917), *MdNPR5a* (KU166921), *MdNPR5b* (KU166920), *MdNPR6* (KU166922), *MdNPR7* (KU166918), and *MdNPR8* (KU166919). The apple *NPR1* homolog gene sequences from ‘Qinguan’ were displayed in the supplementary Fig. S1~S8.

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