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Genome wide *in-silico* analysis of NPR1 gene family in *Citrus reticulata* and its comparison with *Arabidopsis*

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Abstract

Nonexpresser of pathogenesis-related proteins 1 (NPR1), and its paralogue are important salicylic acid (SA) receptors that play important roles in plant defense. NPR gene family analysis has not yet been conducted in *C. reticulata*. The CrNPR1-like and AtNPR1-like protein sequences were retrieved from online genome databases and were subjected to various bioinformatics tools. This study presents the first genome-wide identification of NPR1 gene in *C. reticulata*, resulting in 7 family members. Phylogenetic analysis of 7 CrNPR1-like proteins, along with NPR1-related proteins from 15 species revealed that the proteins were grouped into three major clades. The CrNPR1-like genes in the same *Arabidopsis* subfamilies had similar protein domain compositions, number of exons and conserved motifs. All 7 *CrNPR* genes were segmented duplicated, and no tandem duplicate was observed. Transcriptome data revealed note-worthy expression in leaf, fruit and rind patterns of CrNPR1-like genes. Nearly six out of seven *CrNPR,* expressed in leaf infected with *Xylella fastidiosa,* indicates that these tissues and organs contribute to improved defense response against pathogens. These results pave the way for more functional characterization of *NPR1s* in *C. reticulate* and related species.

Key words: Biotic stress, citrus, expression profile, NPR1, phylogenetic analysis, salicylic acid

Introduction

The mandarin orange (*C. reticulata*), also known as the tangerine or mandarin belongs to family, *Rutaceae*. It is a diploid plant with a chromosome number 2n=2x=18. *C. reticulata*, is generally cited as the ancestral species of cultivated mandarins (Swingle *et al.,* 1967). It is a small, easily peeled, sweet fruit that has a high nutritional importance (Wu *et al.,* 2018) and is a major commercial fruit crop. Satsuma mandarin and Clementine's mandarin are its two commercial varieties. Plant disease in citrus is the major cause of yield decline. Several fungal pathogens attack citrus such as *Colletotrichum acutatum* which causes Post bloom fruit drop; *Alternaria alternata* which causes Alternaria brown spot; *Elsinoe fawsettii* and *E. australis* which causes scab diseases; *Diaporthe citri* which causes melanose; and *Mycosphaerella citri* which causes greasy spot (Timmer *et al.,* 2004). To combat pathogens such as bacteria, oomycetes, viruses and fungi, plants have developed a complex natural immunity. Salicylic acid (SA) mediated signaling pathway is necessary for defense against biotrophic and hemi-biotrophic pathogens (Glazebrook, 2005; Zhang *et al.,* 2019). Nonexpressor of Pathogenesis-Related Proteins 1 (NPR1), and its paralogues NPR3 and NPR4, are true salicylic acid (SA) receptors that operate as vital regulators in defense response known as systemic acquired resistance which is induced by pathogens and is mediated by SA (Ding, 2018; Fu, 2012; Manohar, 2015; Wu *et al.,* 2012). Hence, to understand the immune mechanism of *C. reticulata* it is important to identify and analyze NPR1-like proteins essential in the SA dependent defense response.

Plants are naturally exposed to microorganism like bacteria, fungi, viruses and nematodes. These microorganisms become pathogens when they invade the host cell, proliferate, grab nutrients and damage host cells. In fact, pathogens approaching to enter the cellular cytosol must initially overcome the first layer of plant immune system - the PAMP-triggered immunity (PTI). The receptors present on the cell membrane activate PTI responses by detection of PAMPs, such as fungal chitin, bacterial flagellin (flg22), and lipopolysaccharides (LPS) (Boutrot, 2017; Couto, 2016; Zipfel *et al.,* 2004). However, many microbial pathogens often depress the effect of PTI and lead to effector-triggered susceptibility (ETS) (Deller, 2011; Faris, 2010; Kim *et al.,* 2010). During evolution, plants have developed the second layer of local induced resistance, termed effector-triggered immunity (ETI) (Deller *et al.,* 2011). Plant intracellular sensors encoded by resistance (*R*) genes elicit ETI responses by recognizing these attacker-specific effectors. The *R* gene-mediated defenses confer strong resistance and effectively restrict the growth of pathogens *via.* programmed cell death (PCD), designated as hypersensitive response (HR). This local immune response leads to biosynthesis and accumulation of plant defense hormone SA both at infection sites and in distal uninfected tissues, and then deployment of systemic acquired resistance (SAR) after HR in the whole plant. SAR confers a broad-spectrum, long-lasting, and systemic resistance to secondary infections, that is characterized by expression of many anti-microbial pathogenesis-related (*PR*) genes throughout plant's tissues (Olate, 2018; Wang *et* al., 2016).

Mutant screening is a sole recessive mutation of *Arabidopsis thaliana* in the mid-1990s. It displayed increased disease

susceptibility and eliminated SAR-related gene expression induced by SA- or its analog, called AtNPR1 (Cao *et al.,* 1994), and also called AtNIM1 (Delaney *et al.,* 1995) and AtSAI1 (Shah *et al.,* 1997). *Arabidopsis* NPR1 protein plays a crucial role in systemic acquired resistance mediated by salicylic acid (SA), and is also involved in induced systemic resistance triggered by rhizobacterium (Pieterse *et al.,* 1998), crosstalk inhibition of defense responses mediated by Jasmonic acid (Spoel *et* al., 2008) and cold acclimation (Olate *et al.,* 2018). AtNPR1 has a molecular structure that contains two domains, N-terminal BTB/POZ and central ANK repeat, that participate in protein-protein interactions and are conserved (Boyle, 2009; Rochon *et al.,* 2006). NPR1-like gene family has six paralogues in the *Arabidopsi*s genome (Initiative, 2000), known as AtNPR2, AtNPR3, AtNPR4, Arabidopsis BLADE-ON-PETIOLE2 (AtBOP2; also named AtNPR5), and AtBOP1 (also named AtNPR6) (Hepworth, 2005; Liu, 2005; Norberg *et al.,* 2005). Phylogenetic analysis shows that AtNPR1-like gene family is divided into three functionally separate clades. Two family members are in each of the three clades and have functional redundancy. AtNPR1 and AtNPR2, in the first clade, are SA receptors and act as transcriptional co-activators in plant defense (Castello, 2018; Ding *et al.,* 2018). AtNPR3 and AtNPR4, in the second clade, are also SA receptors and serve as transcriptional co-repressors in plant immune mechanism (Ding, 2018; Zhang *et al.,* 2006). AtBOP1 and AtBOP2, in the third clade, required in plant growth and development (Hepworth, 2005; Norberg *et* al., 2005).

In the absence of pathogen attack, intracellular SA concentration is low and the NPR1 protein resides predominantly in the cytoplasm as an inactive oligomer formed *via.* intermolecular disulfide bonds (Backer *et al.,* 2019). Furthermore, NPR3 and NPR4 are transcriptional co-repressors and interact with TGA (TGACG motif-binding factor) transcription factors (Zhang *et al.,* 2006) to suppress transcription of SA-responsive genes in the nucleus (Ding *et al.,* 2018). In the event of pathogen challenge, intracellular SA content becomes high quickly, resulting in conformational change of NPR1 from oligomer to monomer (Tada *et al.,* 2008). The monomeric NPR1 is then transferred into the nucleus mediated by its C-terminal bipartite nuclear localization signal (NLS) (Kinkema *et al.,* 2000). There, NPR1 binds SA and interacts with the same subset of TGAs to activate its transcriptional co-activator function (Ding *et al.,* 2018). Meanwhile, NPR1 recruits CDK8 (cyclin-dependent kinase 8) and WRKY (W-box-binding factor) transcription factors to the NPR1 promoter to positively regulate its own expression (Chen *et al.,* 2019). Moreover, SA-binding to NPR3 and NPR4 eliminates their transcriptional co-repressor activity on TGAs (Ding *et al.,* 2018). This allows TGAs to turn on defense-related gene expression and activates defense response. In addition to pathogen invasion, exogenous application of SA or its analogs (BTH; benzothiodiazole and INA; 2,6-dichloroisonicotinic acid) could also induce the resistance mechanism in plants (An, 2011; Tripathi *et al.,* 2019). Together, NPR1, and its paralogues NPR3 and NPR4, are all SA receptors through an antagonistic manner to finely regulate plant immune response dependent on distinct threshold levels of SA (Ding *et al.,* 2018).

On the basis of above research, a systematic identification and analysis of NPR1-like family in *C. reticulata* is required. This study has been made possible due to a reference genome sequence published recently (Wang *et al.,* 2018). Here, the NPR1-like family was identified in *C. reticulata* genomes. These reputed NPR1-like genes were analyzed in detail, including molecular characterization, phylogenetic classification, gene structures, protein domain compositions, conserved motifs and cis-regulatory elements. In addition, using publicly available *C. reticulata* RNA-sequencing (RNA-seq) datasets, the expression pattern of CrNPR1-like genes in various tissues/organs and under biotic stress conditions was also studied.

Material and methods

Sequence data source: The NPR1-like protein sequences of *Oryza sativa* (Os), *Lilium* (LhSor), *Gossypium hirsutum* (Gh), *Carica papaya* (Cp), *Glycine max* (g), *Vitis vinifera* (Vv), *Zea mays* (Zm), *Nicotiana tabacum* (Nt), *Brassica juncea* (Bj), *T. dicoccoide* (w), *Gladiolus hybridus* (Gh), *Brachypodium distachyon* (Abd Elwahaab), *Arabidopsis thaliana* (At), *Malus domestica* (Md), *Pyrus pyrifolia* (Dey), were taken from the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/). The genome sequences and NPR1-related protein sequences of *C. reticulata* were acquired from Citrus genome database (https://www.citrusgenomedb.org/blast/report/PytzEytKm).

Identification **of NPR1-like genes:** A BLASTP using an *Arabidopsis,* rice, papaya and wheat NPR1 like protein sequence was used as a query for the identification of *NPR* gene in the *C. reticulata* in proteome database (https://www.citrusgenomedb.org/blast/report/PytzEytKm). Then to confirm the presence of *NPR like* domains, retrieved amino acid sequences were subjected to searches at the SMART (http://smart.embl-heidelberg.de/) (Letunic, 2006; Letunic *et al.,* 2004), and NCBI CDD (Conserved Domain Database) (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (Marchler-Bauer, 2015; Marchler-Bauer *et al.,* 2013) with the default parameters. The proteins lacking real ANK repeats in the central region and N-terminal BTB/POZ domain were identified and excluded. Overall, 7 putative NPR1-like sequences were detected in the *C. reticulata* genome. Gene structure (Exon-Intron) was visualized in the GSDS (Gene Structure Display Server) (http://gsds.cbi.pku.edu.cn/) (Guo, 2007; Hu *et al.,* 2015) by loading both *NPR* coding sequences and corresponding genomic sequences. The protein length (amino acid residues), molecular weight, and theoretical pI of *CrNPR* proteins were predicted using ProtParam tool (http://web.expasy.org/protparam/) (Garg *et al.,* 2016). The information for gene IDs, chromosomal position, sequence of gene and protein, were retrieved from *C. reticulata* proteome database at (https://www.citrusgenomedb.org/blast/report/PytzEytKm). These *CrNPR* genes were renamed according to the order of their physical position.

Subcellular localization analysis, promoter prediction and conserved motifs recognition: The nuclear localization signals in *C. reticulata NPR* proteins were predicted through an online server NLSdb (https://rostlab.org/services/nlsdb/) (Bernhofer *et al.,* 2018). Subcellular localization of *CrNPR* was predicted using the online tool WoLF PSORT (https://wolfpsort.hgc.jp/) (Horton *et al.,* 2006). For analysis of promoter region, a sequence of 1000-bp upstream was retrieved from initiation codon of the putative CrNPR genes. Plant Care database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was then used to predict *cis*-regulatory elements in these sequences and validated in the PLACE databases (http://www.dna.affrc.go.jp/PLACE/). Multiple

EM for Motif Elicitation (MEME) program (http://meme.nbcr.net/meme/) was used to analyze with the concluded protein sequences of the *CrNPR* with maximum number of motifs set as 10. The minimum 6 width of motif and maximum 50 width of motif were set as default values along with other factors.

Multiple sequence alignment and phylogenetic tree construction: The amino acid sequences of *CrNPR* proteins were aligned using Clustal W version 2.1 (Thompson *et al.,* 2002) and phylogeny through MEGA v x.0 (http://www.megasoftware.net) program with the neighbor joining (NJ) and bootstrapping set at 1000 replications. There were 7 CrNPR, 6 *Arabidopsis (At)*, 4 *Oryza sativa (Os),* 2 *Glycine max* (g) 1 *Lilium* (LhSor), 1 *Gossypium hirsutum* (Gh), 1 *Carica papaya* (Cp), *1 Vitis vinifera* (Vv), 1 *Zea mays* (Zm), 1 *Nicotiana tabacum* (Nt), 1 *Brassica juncea* (Bj), 1 *T.dicoccoide* (w), 1 *Gladiolus hybridus* (Gh), 1 *Brachypodium distachyon* (Abd Elwahaab), 1 *Malus domestica* (Md), 1 *Pyrus pyrifolia* (Dey) and 1 *Ipomoea batatas* (Ib) NPR protein sequences were used for phylogenetic analysis. Two genes from distinct species on adjacent branches of same clade in the phylogenetic tree were defined as orthologs.

Gene duplication, chromosome location and calculation of nonsynonymous (Ka) and synonymous (Ks) substitution rates: Gene pairs of *CrNPR1* were generated using the phylogenetic, motif and domain analysis data and used to compute the Ka and Ks substitution rates with tbtools (Chen *et al.,* 2020). Gene pairs along with CDS sequence and protein sequence of NPR1 like genes of *C. reticulata* were used. The molecular evolutionary rates of every single gene pair were ascertained by evaluating Ka/Ks ratio. Usually, Ka/Ks < 1 signifies purifying selection; Ka/Ks = 1 signifies neutral selection; and Ka/Ks > 1 signifies positive selection. The divergence time of these gene pairs was measured approximately by applying the formula "t = Ks/2r", with r (1.5 × 10^-8) denoting neutral substitution. MG2C (http://mg2c.iask.in/mg2c_v2.0/) was utilized to map the CrNPR1-like genes on scaffolds to display their distribution. The duplicated genes were marked with line joining on the map.

Percent amino acid identity in CrNPR Gene: Percent amino acid identity between sequences was determined by the MegAlign program of the Lasergene software package version 7.2.1 (Burland *et al.,* 2000).

Expression analysis: PlantGDB (http://www.plantgdb.org/) expressed sequence tag (EST) in-silico investigation was performed from the identified CrNPR1-like genes to estimate their expression status in various important *C. reticulata* plant tissues and organs. The CDS sequences of each NPR1 gene were used to derive the EST information using Blastn search with the default parameter and e-value=1e−04. Finally, a heatmap using tbtool (Chen *et al.,* 2020) was created to characterize the particular NPR gene expression into various tissues and organs in *C. reticulata.*

CrNPR FPKM gene expression data from a staygreen mutant of citrus and its wild type was retrieved from NCBI gene expression omnibus (accession number is GSE94810). A heatmap of the FPKM values was made using tbtools (Chen *et al.,* 2020).

miRNA expression dataset in *C. reticulata*: We obtained microRNA (miRNA) sequences of *C. reticulata* from NCBI Gene Expression Omnibus (accession number is GSE116095) and published articles. NPR genes targeted by miRNAs were predicted by searching their CDS regions for complementary sequences using the psRNATarget server with default parameters, (E) = 4.0 (http://plantgrn.noble.org/psRNATarget/home).

Results and discussion

Identification, phylogeny and characterization of NPR1-Like genes in *C. reticulata*: The 7 putative NPR1-like genes in *C. reticulata* genome were distinguished using bioinformatic tools (Table 1). Phylogenetic grouping can possibly provide a source for comprehending functional diversity of the NPR1-like family since individual phylogenetic analysis fails to satisfactorily perform functional annotation (Abd Elwahaab, 2019; Drillon *et al.,* 2020). A 32 NPR sequence unrooted phylogenetic tree (7 CrNPR, 6 atNPR, 4 *OSNPR,* 2 *GmNPR,* 1 LhSorNPR, 1 GhNPR, 1 CpNPR, *1* VvNPR, 1 ZmNPR, 1 NtNPR, 1 BjNPR, 1TdNPR, 1 *GhNPR*, 1 BdNPR, 1 MdNPR, 1 PpNPR and 1 IbNPR) was constructed using Mega-X (Kumar *et al.,* 2016). Theses NPRs of six monocots and nine dicots from published materials were acquired through molecular cloning techniques (Ali, 2017; Olate *et al.,* 2018). The results showed that the NPR1-like proteins were grouped into three major clades; Clade I (AtNPR3/4 subfamily) containing OsNPR2/3, GmNPR1, MdNPR1 etc., Clade II (AtBOP1/2 subfamily) containing OsNPR5 AND CrNPR8 and clade III (AtNPR1/2 subfamily) containing OsNPR1 Wnpr1 etc. Clade I contained CrNPR2, CrNPR3, CrNPR4, CrNPR5 and CrNPR6 (Fig. 1).

Table 1. Information about 7 non-redundant NPR1-like genes discovered from the genome of *C. reticulata* Notes: AA, amino acid sequence length; MW, molecular weight; pI, isoelectric point

NPR1 Gene	Accession Number		Scaffold Location	Scaffold	AA	Length of mRNA	Intron	MW	pI	LOP
	CitrusGDB	PLAZA DB								
CrNPR1	MSYJ077460_1	Ciclev10007832m.g	345601..349806	scaffold85959_cov71	585	4206	3	65789.71	6.31	cyto, nucl
CrNPR2	MSYJ131960_1	Ciclev10017873m.g	900842..904108	scaffold86042_cov89	587	3267	3	65938.7	5.7	nucl, cyto_nucl, cyto
CrNPR3	MSYJ281930_1	Ciclev10033533m.g	1546236..1555299	scaffold85940_cov86	592	9064	3	65764.77	5.9	nucl, cyto_nucl, chlo, cyto, plas
CrNPR4	MSYJ281950_1	Ciclev10031343m.g	1596698..1666125	scaffold85940_cov86	565	69428	7	63352.37	5.8	chlo
CrNPR5	MSYJ281960_1	Ciclev10031627m.g	1668069..1688336	scaffold85940_cov86	800	20268	11	89049.19	7.45	E.R., E.R._plas, plas, cyto, chlo, nucl, mito
CrNPR6	MSYJ281940_1	Ciclev10031258m.g	1562898..1565135	scaffold85940_cov86	416	2238	1	47346.89	8.74	chlo, mito
CrNPR8	MSYJ287170_1	Ciclev10031432m.g	452682..455352	scaffold86089_cov86	448	2671	2	49421.35	6.28	E.R., chlo, nucl, vacu, cyto, mito

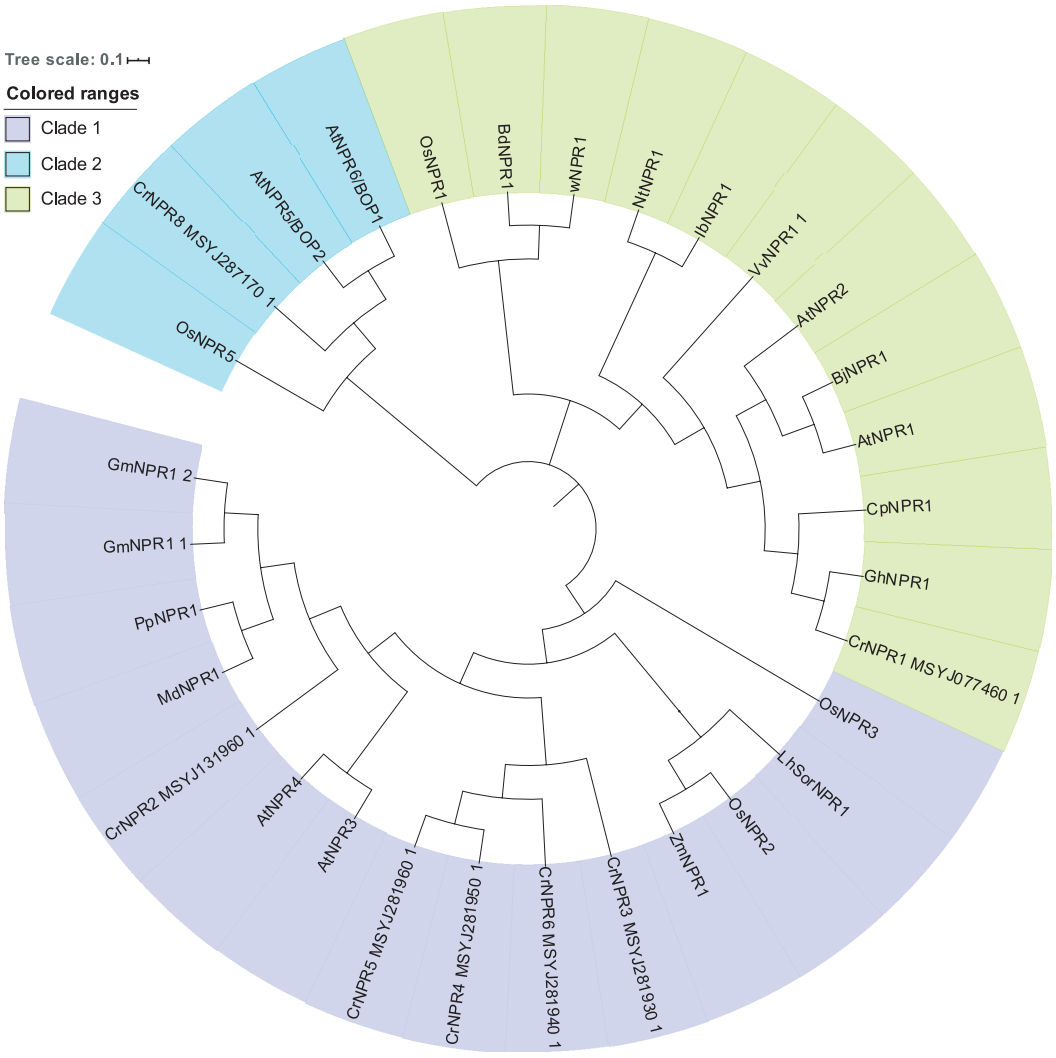

Fig. 1. Polygenetic tree of NPR1 like proteins in *C. reticulata* and its homologous in *O. sativa* (Os), *Lilium* (LhSor), *G. hirsutum* (Gh), *C. papaya* (Cp), *G. max* (g), *V. vinifera* (Vv), *Z. mays* (Zm), *N. tabacum* (Nt), *B. juncea* (Bj), *T. dicoccoide* (w), *G. hybridus* (Gh), *B. distachyon* (Abd Elwahaab), *A. thaliana* (At), *M. domestica* (Mp), and *P. pyrifolia* (Dey).

All of the clade I NPR1-LIKE genes except CrNPR2 are on the same scaffold that is scaffold85940_cov86. Clade II contained CrNPR8 which is on scaffold86089_cov86 and clade III contained CrNPR1 which is on scaffold85959_cov71 (Figure S1). CrNPR proteins had a sequence length that ranged from 800 (CrNPR5) to 416 (CrNPR6) amino acids. The average isoelectric point of CrNPR members was a weakly acidic value of 6.6, generally it varied from 5.7 (CrNPR2) to 8.74 (CrNPR6). The molecular weight ranged from 89049.19 (CrNPR5) to 47346.89 (CrNPR6) with an average value of 63808.99714 (Table 1).

Sequence and structural analysis of CrNPR1-like genes and proteins: Utilizing GSDS and NCBI-CDD the structural feature and sequence composition were analyzed to further study the potential functions of CrNPR1-like genes. The exon-intron structure of CrNPR1-like genes was similar to the corresponding AtNPR1-LIKE genes (Fig. 2). Clade III, CrNPR1 and AtNPR1/2 had 4 exons and 3 introns. Although AtNPR3/4 in clade I also had 4 exons and 3 introns only CrNPR2 and CrNPR3 had similar exon-intron structure. The rest of NPR1-like genes had a different number of exons. Moreover, in clade II the exon-intron structure of CrNPR8 is different from its complementary AtBOP1/2 (Fig. 2). Furthermore, *CrNPR6* and AtNPR5-6 have similar exon/intron structure pattern while CrNPR4 and CrNPR5 contained extra intron and exon which could either be an assembly error or a unique aspect of these gene.

Furthermore, only 7 CrNPR1-like genes contained central ankyrin and N-terminal BTB/POZ domains like AtNPR1 as demonstrated by the protein composition (Fig. 3). In addition, all NPR1-LIKE genes except NPR8 included the NPR1-like C-terminal region that was vital for AtNPR1 activity (Rochon *et* al., 2006).

The Motif analysis showed that Motifs 4, 3, 5, 2, 1 and 6 were in all of the CrNPR and AtNPR proteins (Fig. 4). Motif 1 and

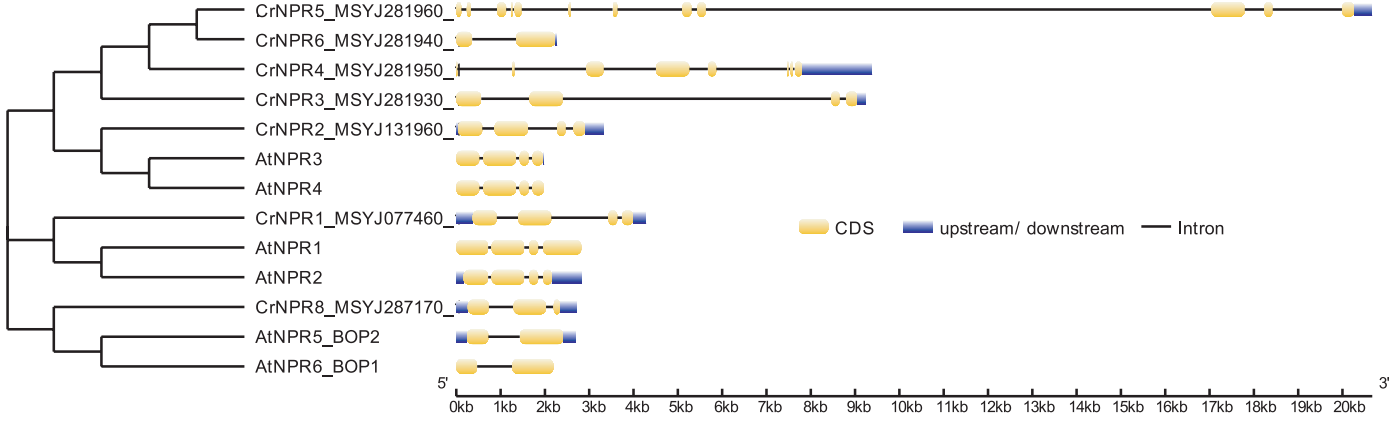

Fig. 2. Phylogenetic relationship and gene structure of NPR1 genes from *C. reticulata* and *A. thaliana*. The phylogenetic tree was constructed using full length sequences of CrNPR1-like genes and AtNPR1-like genes. Yellow boxes indicate exons; and black lines indicate introns

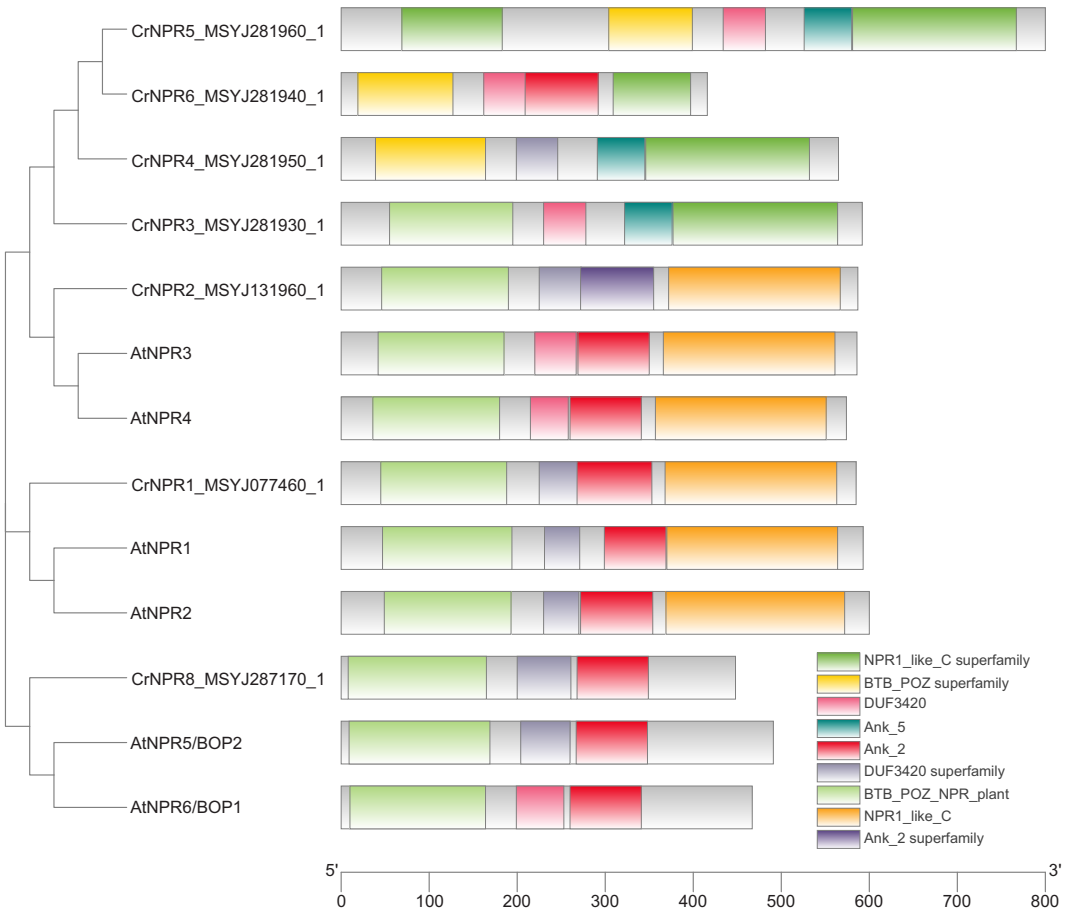

Fig. 3. The domain patterns of putative NPR1-Llike genes in *C. reticulata* and *A. thaliana* interlinked with phylogenetic tree for better understanding

Motif 2 are ankyrin repeats, motif 4 is BTB/POZ domains, motif 5 is DUF3420 and motif 6 is NPR1_like_C (Table S1). The information of motif 3 was not found. On analyzing the motifs in NPR1-like proteins in *A. thaliana* and then comparing them with the motifs in CrNPR proteins it was found that they had the same kind of motifs conserved which made them putative NPR1-like proteins (Fig. 5).

Analysis of cis-regulatory elements in the promoter sequences of CrNPR1-like genes and sub-cellular localization analysis: By interacting with transcription factors cis-regulatory elements control the expression of plant genes (Priest *et* al., 2009). So, to further understand the regulatory expression, potential cis-regulatory elements in the promoter region of CrNPR1-LIKE genes were identified. For SA-responsive cis-regulatory identification 1000-bp upstream promoter sequences of CrNPR1-like genes were selected and submitted to the Plant CARE online service (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). The promoter sequences of only three CrNPR1-like genes, CrNPR1, CrNPR2 and CrNPR8, were found. The analysis showed that majority of the CrNPR1-like gene's promoters contained CAAT box and is a hormone related regulatory elements. MYB which is involved in the ABA response was also in most of the promoter sequences. Only CrNPR1 had as-1 (The activation sequence-1) element (TGACG) required in transcription activation of various SA-regulated PR genes (Fig. 2).

In the sub-cellular localization analysis, it was revealed that cytoplasm, nucleus and chloroplast had the higher number of CrNPR1-like genes. CrNPR1, CrNPR2 and CrNPR3 were in higher number in the cytoplasm and nucleus whereas CrNPR4, CrNPR6 and CrNPR8 were in higher number in chloroplast. CrNPR5 was in a higher number in cytoplasm while its number in nucleus and chloroplast was the same. (Fig. 6).

Calculation of nonsynonymous (Ka) and synonymous (Ks) substitution rates and Ka/Ks ratios of NPR1 gene in *C. reticulata*: Tbtools program was used to determine the Ks and Ka value together with the Ka /Ks ratios. Then the estimate date of gene duplication events was calculated. The *Ka* corresponds to the number of nonsynonymous substitutions per nonsynonymous site while *Ks* is the number of synonymous substitutions per synonymous site and *Ka* /*Ks* denotes the ratios of nonsynonymous (*Ka*) to synonymous (*Ks*) mutation. The paralogous groups had no tandem gene duplication. The duplication date for segmental duplications of the paralogous pairs ranged from 152.79 Mya ($K s = 4.58$) for paralogous group *CrNPR1/CrNPR2* to 7.18 Mya ($K s = 0.21$) for *CrNPR4/CrNPR5*. In 6 pairs of paralogous groups the *Ka* /*Ks* ratio was more than 0.3 suggesting the possibility of existence of significant functional divergence after duplication events and 9 pairs had *KA* /*K s* ratio <0.3 signifying no functional divergence (Fig. 7).

Percent amino acid identity: Percent amino acid identity between the predicted amino acid sequences of CrNPR1 homologues and the Arabidopsis NPR1 homologues ranged from 81.2 % (CrNPR8 vs AtNPR5) to 20.8 % (CrNPR5 vs AtNPR6), whereas amino acid identity among the *C. reticulata* NPR1 homologues ranged from 20.5 % (CrNPR5 vs CrNPR8) and 75.6 % (CrNPR4 vs CrNPR5). A similar percent amino acid identity was found among Arabidopsis NPR1 sequences (Yuan, 2007; Zhong *et al.,* 2015) (Table 2).

Expression analysis of CrNPR1-like genes in various tissues/organs: In order to further study the characteristics and functions of the CrNPR gene family, the expression patterns of CrNPR gene family members in different tissues were analyzed by EST analysis at Plant genome dB (Fig. 8) and CrNPR FPKM gene expression data from a staygreen mutant of citrus (Alos *et al.,* 2008) and its wild type NCBI GEO (accession number is GSE94810). A heatmap of the FPKM values was made using tbtools (Fig. 9) (Chen *et al.,* 2020). Expressed sequence tags (EST) data analysis revealed that several NPR1 genes were expressed in numerous important tissues and organs in *C. reticulata*. It suggested that NPR gene families showed substantial and noteworthy expression

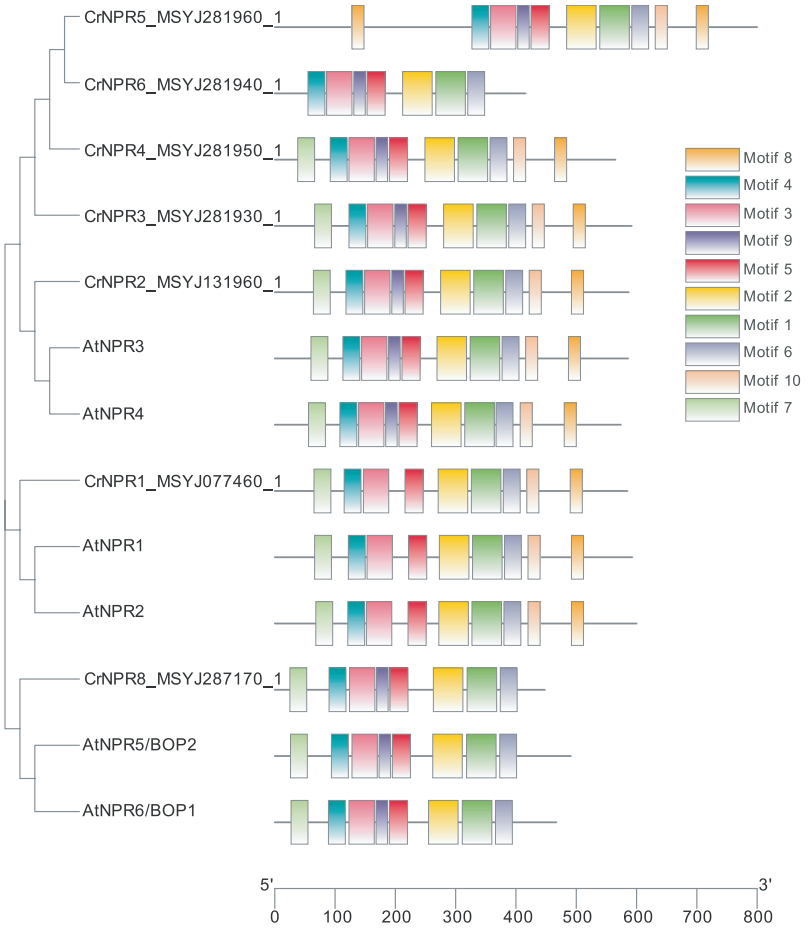

Fig. 4. The distribution of 10 motifs presents in 7 NPR proteins of *C. reticulata* by using MEME version 4.9.0 and interlinking it with CrNPR phylogenetic tree to develop a good understanding of their association

in leaf, fruit and rind (Fig. 8). It is however observed that almost all members of CrNPR1-like gene family exhibited their expression at least in one tissue or organ. However, CrNPR8 was not expressed in any tissue or organ in *C. reticulata*. Nearly 85 % NPR genes of all 7, expressed in leaf infected with *Xylella fastidiosa* (Souza *et al.,* 2007) (Fig. 8). This surely indicates that these tissues and organs contribute to improved defense response against bacterial pathogens. Among the NPR1-like gene members, CrNPR3, CrNPR4, CrNPR5, CrNPR6 exhibited in leaf (*X. fastidiosa*), fruit (Development stadium), Clementine Mandarin albedo/ rind separation and leaf (greenhouse). CrNPR1 only had leaf (*X. fastidiosa*) expression. The results suggest that SAR, the defense response, mainly occurs in leaf and fruit. It takes place to a lesser extent in the rind.

Since high-throughput RNA sequencing and gene expression analyses have been performed on many citrus tissues at various developmental stages, publicly-available RNA-Seq data is thought to be useful resources for studying gene expression profiles. Distinct transcript abundance patterns were readily identifiable in the RNA-Seq dataset at NCBI. The heatmap of expression profiles of CrNPR1-like genes in NCBI GEO (accession number is GSE94810) are presented in Fig. 9. This data is from the flavedo of the citrus wild type and stay green mutant fruit that were collected at 210 days after flowering and 30 days after storage for RNA extraction and analysis. Fruit showed that there was high gene expression in CrNPR3, CrNPR4, CrNPR6 and CrNPR8. CrNPR5 had no gene expression. Overall,

Clade	Gene Id	Motifs										
A	AtNPR1	BTB7		BTB4	3		5	AR2	AR1	NLC6	10	8
	AtNPR2	BTB7		BTB4	3		5	AR2	AR1	NLC6	10	8
	CrNPR1	BTB7		BTB4	3		5	AR2	AR1	NLC6	10	8
B	CrNPR8	BTB7		BTB4	3	9	5	AR2	AR1	NLC6		
	AtNPR5	BTB7		BTB4	3	9	5	AR2	AR1	NLC6		
	AtNPR6	BTB7		BTB4	3	9	5	AR2	AR1	NLC6		
C	AtNPR3	BTB7		BTB4	3	9	5	AR2	AR1	NLC6	10	8
	AtNPR4	BTB7		BTB4	3	9	5	AR2	AR1	NLC6	10	8
	CrNPR2	BTB7		BTB4	3	9	5	AR2	AR1	NLC6	10	8
	CrNPR3	BTB7		BTB4	3	9	5	AR2	AR1	NLC6	10	8
	CrNPR4	BTB7		BTB4	3	9	5	AR2	AR1	NLC6	10	8
	CrNPR5		8	BTB4	3	9	5	AR2	AR1	NLC6	10	8
	CrNPR6			BTB4	3	9	5	AR2	AR1	NLC6		

Fig. 5. The heatmap shows the comparison of the motifs pattern of NPR1-like proteins in *C. reticulata* and *A. thaliana*

Table 2. Percent similarity of CrNPR 1-like protein sequence of *C. reticulata* and *A.thaliana*.

CrNPR1	CrNPR2	CrNPR3	CrNPR4	CrNPR5	CrNPR6	CrNPR8	AtNPR1	AtNPR2	AtNPR3	AtNPR4	AtNPR5	AtNPR6	
***	44.1	38.8	30.3	30.1	34.1	24.8	53.5	51.6	40.5	40.6	22.2	24.8	CrNPR1
	***	59.5	46.7	42.8	49.8	27.9	38.8	36.8	59.7	57.8	23.8	27.2	CrNPR2
		***	68.3	65.4	66.3	29	32.3	31.1	46.9	49.8	25.7	26.1	CrNPR3
			***	75.6	68	27	28.7	28.1	38.4	40.2	23.8	25.7	CrNPR4
				***	66.1	20.5	27.7	26.2	36.3	38	19.8	20.8	CrNPR5
					***	22.1	31	30.5	41.3	43.3	20.9	21.4	CrNPR6
						***	24.8	21.2	25.9	25.7	81.2	76.8	CrNPR8
							***	61.4	35.7	36.4	20	21.2	AtNPR1
								***	35.8	37.5	18.9	22.9	AtNPR2
									***	71.8	24.2	25.1	AtNPR3
										***	24.4	25.7	AtNPR4
											***	82.9	AtNPR5
												***	AtNPR

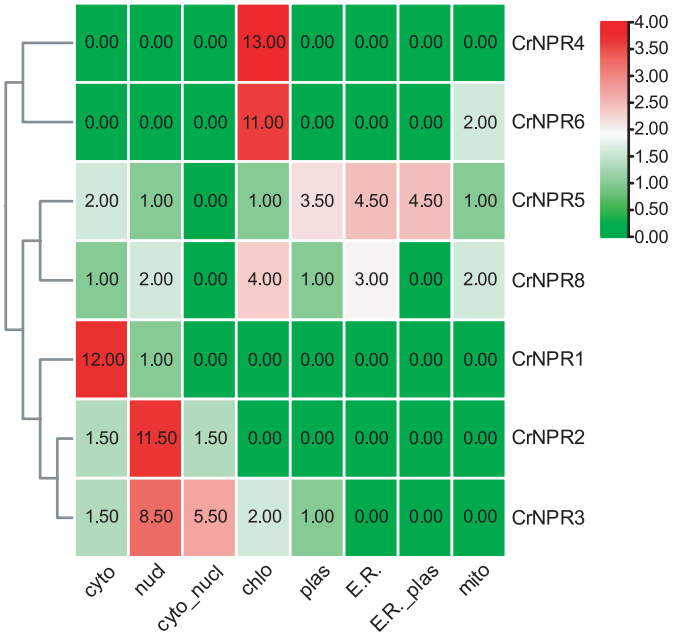

Fig. 6. The sub-cellular presence of putative NPR1-Llike genes in *C. reticulata* interlinked with phylogenetic tree for better understanding of different gene function and similarity

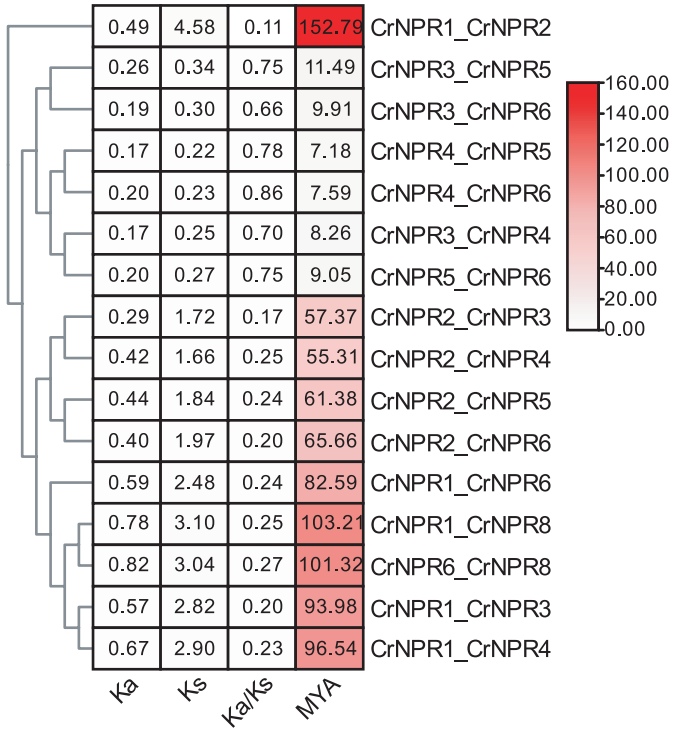

Fig. 7. Time of gene duplication estimated for different paralogous pairs of *C. reticulata* NPR1 like genes on the basis of Ks and Ka values. Analyses were conducted using the tbtools (Chen *et al*., 2020)

the highest gene expression was in CrNPR4 whereas the lowest gene expression was in CrNPR1 (Fig. 9).

miRNA expression dataset in *C. reticulata*: In the analysis 7 miRNA were identified which were related to 6 CrNPR1-like genes. No miRNA target was found for CrNPR2. Each of the CrNPR1-like sequences with identified miRNAs had at least 1 matched sequence. While CrNPR4 had 4 miRNA sequence matches in its sequence. The sequences and other details of the above mentioned miRNAs is given in Table 3.

NPR like proteins is involved in different defense and signaling pathways in different plant species (Ali, 2017; Olate *et al.,* 2018).

This study is the first genome wide investigation of the *NPR1*-like gene family in *C. reticulata*, comparable to other plant species, like *O. sativa* (Yuan *et al.,* 2007) and *A. thaliana* (Hepworth *et al.,* 2005) which have 4 and 6 NPRs, respectively. This analysis sets the foundation for further functional characterization of the NPR1-like protein family in *C. reticulata*. The phylogenetic tree of the seven CrNPRs showed that NPR-like genes were unevenly distributed in 3 clades with majority of the genes in clade I (Fig. 1) (Table 4).

The *C. reticulata* genome contains seven identifiable *NPR1*-like genes; these sequences share similar gene structures and protein sequence identities as well as conserved domains and motifs present in *Arabidopsis NPR1*-like sequences (Backer *et al.,* 2015).

It shows that two main clades, NPR and BOP, arise from a progenitor gene of NPR1-like family through duplication and differentiation (Liu *et* al., 2005). Then, the ancestor NPR gene might go through a second series of duplication event resulting in the NPR1/2 and the NPR3/4 clades. The ancient duplication events leading to functional divergence of NPR1-like genes probably happened before the monocot-dicot split because the aforementioned 6 monocots and 9 dicots have at least one member in each clade (Shia *et al.,* 2013). The current state of each clade may have been achieved by another series of duplication event after the monocot-dicot split. For example, six NPRs (AtNPR1/AtNPR2, AtNPR3/AtNPR4, and AtBOP1/AtBOP2) in *Arabidopsis* and seven NPRs (CrNPR1, CrNPR2/CrNPR3/NPR4/CrNPR5/CrNPR6 and CrNPR8) in *C. reticulata*.

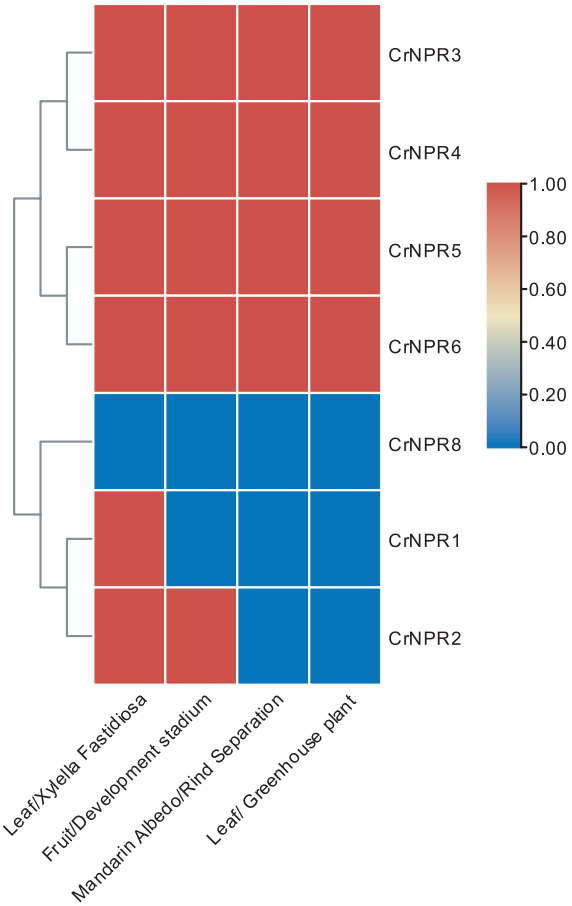

Fig. 8. The heat map shows the EST expression profile of the CrNPR1-like genes in different organs in *C. reticulata*. The x-axis represents names of the four parts of *C. reticulata*, and the y-axis represents different CrNPR1-like genes. The expression levels of CrNPR genes are revealed by different colors, which increase from blue to red

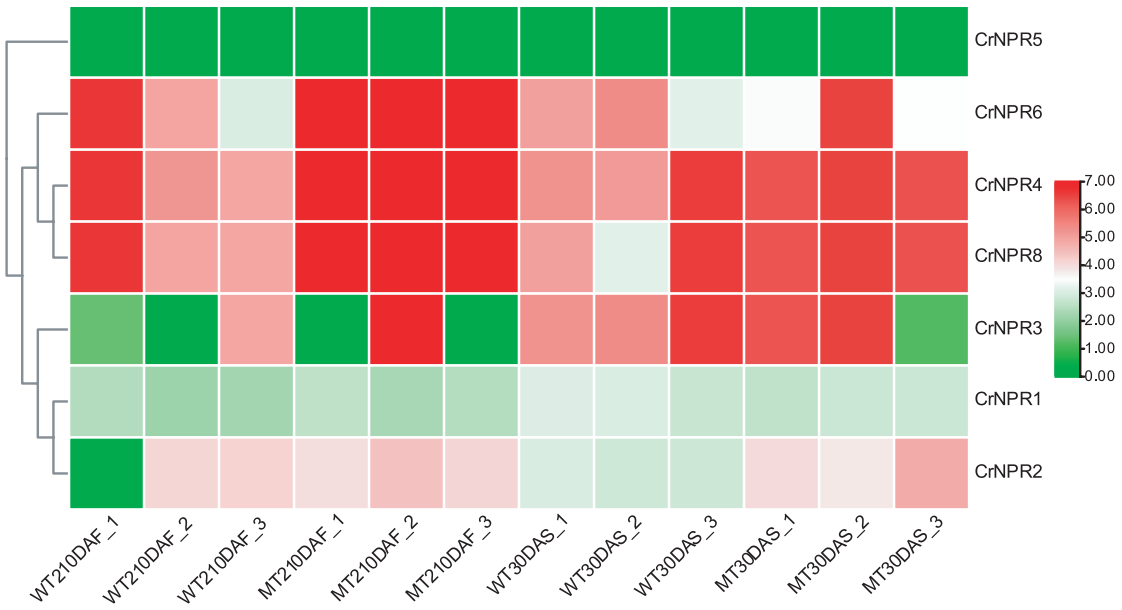

Fig. 9. The heatmap expression profile of *C. reticulata* NPR like genes across wild type and stay green mutant oranges. X-axis shows 12 samples and the putative CrNPR1-like genes on the y-axis. The FPKM values are revealed by different colors and increase from green to red. The source of the samples is as follow: WT210DAF (Wild type 210 Day after flowering), MT210DAF:(Mutant 210 Day after flowering), WT210DAS (Wild type 210 Day after storage) and MT210DAS (Wild type 210 Day after storage)

Table 3. miRNA targets prediction of CrNPRs

miRNA id	Gene Id	Ref_Seq	Alignment	RPM
orange-MIR473-81	CrNPR5	ACUCUCCCUCAAGGGCUUCGC	miRNA 21…1 Target 1817…1837	277.2611
orange-MIR473-81	CrNPR4	ACUCUCCCUCAAGGGCUUCGC	miRNA 21…1 Target 1112…1132	277.2611
orange-MIR477b-97	CrNPR5	CUCUCCCUCAAGGGCUUCUCU	miRNA 21…1 Target 1816…1836	197.6596
orange-MIR477b-97	CrNPR4	CUCUCCCUCAAGGGCUUCUCU	miRNA 21…1 Target 111…1131	197.6596
orange-MIR166g-15	CrNPR1	GGAAUGUUGUCUGGCUCGAGG	miRNA 21…1 Target 570…590	220.4029
orange-MIR390a-4	CrNPR4	AAGCUCAGGAGGGAUAGCGCC	miRNA 21…1 Target 454…474	253.2773
orange-MIR473-81	CrNPR6	ACUCUCCCUCAAGGGCUUCGC	miRNA 21…1 Target 1001…1021	277.2611
orange-MIR477b-97	CrNPR6	CUCUCCCUCAAGGGCUUCUCU	miRNA 21…1 Target 1000…1020	197.6596
orange-MIR482a-80	CrNPR4	AGUGGGAGCGUGGGGUAAGAAG	miRNA 22…1 Target 1473…1494	25.63786
orange-MIR390a-4	CrNPR5	AAGCUCAGGAGGGAUAGCGCC	miRNA 21…1 Target 1159…1179	253.2773
orange-MIR477b-97	CrNPR3	CUCUCCCUCAAGGGCUUCUCU	miRNA 21…1 Target 1204…1224	197.6596
orange-MIR482b-110	CrNPR8	UCUUGCCCACCCCUCCCAUUCC	miRNA 22…1 Target 631…652	4053.056
orange-MIR535-20	CrNPR1	UGACAACGAGAGAGAGCACGC	miRNA 21…1 Target 1509…1529	10.75136

Additionally, the structural and sequence features of CrNPR also support the phylogenetic analysis. CrNPR3 and CrNPR2 have 3 introns each which is the same number of introns as AtNPR3 and AtNPR4 that are in the same clade. Similarly, NPR1 and AtNPR1/2 on Clade III share similar exon-intron structure. On the other hand, NPRs in clade II have different number of introns. However, all the CrNPR proteins contain Ank and BTB/POZ domains. Only NPR8 in clade II does not harbor NPR-like C terminal region.

Biological functions in a plant are usually reflected by the tissue/organ-specific expression patterns (Barsalobres-Cavallari, 2009; Yang *et al.,* 2013). CrNPRs demonstrated specific expression in the leaf, fruit and rind in the in-silico assessment of RNA-seq experiments. This suggests that they may participate in development and protecting the seed for plant propagation.

CrNPR1-like gene expressions were examined by investigating the expression profiles of CrNPR1-like genes upon biotic stresses. In orange fruit in response to the application of the antagonist all of the CrNPRs were expressed at different FPKMs (Fig. 9).

Table 4. *C. reticulata* NPR gene family distribution among clades and groups based on phylogenetic analysis with Arabidopsis NPR member

Group	Number of NPR Gene		Gene Id	
	At	Cr	At	Cr
A	2	1	AtNPR1, AtNPR2	CrNPR1
B	2	1	AtNPR5, AtNPR6	CrNPR8
C	2	5	AtNPR3, AtNPR4	CrNPR2, CrNPR3, CrNPR4, CrNPR5, CrNPR6

Altogether, these putative NPR1-like genes could be used as the preferred genes to prove their biological functions through molecular experiments in development and defense.

A total of 7 NPR1-like genes were identified from *C. reticulata*. The CrNPR1-like genes were studied in depth, comprising protein domain compositions, molecular characterization, gene structures, phylogenetic classification and conserved motifs, as well as cis-regulatory elements. CrNPR1-like genes displayed particular tissue/organ specific expression patterns based on RNA-seq data. These findings will be beneficial in planning experiments to assess the biological functions and understand the evolutionary relationship of the NPR1-like gene family in *C. reticulata*.

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