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Research Article

In Silico Study of *NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1* (*NPR1*) Sequence in Citrus Associated with Huanglongbing Resistance

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ABSTRACT

The evaluation of citrus resistance to Huanglongbing (HLB) disease is still challenging due to the incapability of the bacteria to be cultured purely in artificial medium, the complexity of inoculation methods, and the long duration required for phenotypic observation. Thus, the use of molecular markers is one of the alternatives to solve this problem. The focuses of this study were to perform *in silico* analysis of the nucleotide variations in *NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1* (*NPR1*) gene sequence among several citrus genotypes whose resistance information to HLB have been known previously and to employ phylogenetic analysis among them. The *NPR1* gene sequences from 20 genotypes which consisted of 14 citrus and six relative genotypes were collected *in silico* from Citrus Genome Database and analyzed using multiple sequence alignment program. A total of six interesting SNPs that could distinguish between susceptible and resistant citrus genotypes were detected in this study. As many as five SNPs were non-synonymous, while only one synonymous SNP that did not cause the amino acid change was identified in this study. The phylogenetic analysis also revealed the separation between susceptible and tolerant/resistant citrus genotypes in two main clusters. The SNPs found in this study are expected to be useful for designing new functional markers as a selection tool in future studies.

Keywords: *in silico*, molecular marker, phylogenetic analysis, selection tool, SNP

Introduction

The evaluation of citrus resistance to Huanglongbing (HLB) disease is still challenging due to the incapability of the bacteria to be cultured purely in artificial medium. In Asian region, the disease is caused by a bacterium namely *Candidatus* Liberibacter asiaticus (*C*Las) that is heat tolerant and disseminated by Asian citrus psyllid (*Diaphorina citri*) or via grafting propagation (Bové, 2006; Cui et al., 2022; Grafton-Cardwell et al., 2013; Jagoueix et al., 1996). The disease promotes several morphological symptoms such as plant height reduction, yellow shoots, blotchy mottled with vein corking leaves, asymmetrical small fruits with poor color and bitter taste, fruits abscission, and less fibrous root (Chen et al., 2016; Nehela & Kiliny, 2020), which is similar to and misunderstood as nutrient deficiency symptoms (Tipu et al., 2021).

Based on genome sequence analysis, *C*Las is classified in Rhizobiaceae family as well as *Rhizobium* and *Agrobacterium* (Duan et al., 2009). However, this bacterium is still difficult to be cultured purely in artificial medium (Li et al., 2016), unlike the other Rhizobiaceae members. Previous study from Davis et al. (2008) reported the success of *C*Las culture using babaco basal medium (BBM), while Sechler et al. (2009) proficiently formulated Liber A medium for *C*Las culture. On the other side, Parker et al. (2014) reported the use of King's B medium enriched with grapefruit commercial juice for *C*Las culture. Another study from Zheng et al. (2024) also reported the success of *C*Las culture using liquid LG medium.

Besides the medium culture, the inoculation methods also played crucial role in citrus resistance evaluation to HLB disease. Previous studies from Lopes and Cifuentes-Arenas (2021) and Zheng et al. (2024) reported the use of psyllid feeding method. Nevertheless, the successful rate of this method was relatively low, approximately 9.4% (Zheng et al., 2024). Kosmiatin et al. (2020) also reported a host-free culture method via *in vitro* inoculation. Another inoculation method is via grafting by using infected scions which grafted to healthy rootstocks (Cui et al., 2022; Wang et al., 2025). However, these inoculation methods are time consuming due to the requirement of 6 to 8 months for subsequent analysis by PCR or qPCR to ensure the bacteria existence in citrus phloem tissue.

A fast and reliable screening method should be developed for citrus resistance evaluation to HLB disease, to help the acceleration of citrus breeding programs. The use of molecular marker is one of the alternatives due to the high polymorphisms to the nucleotide variations level, uninfluenced by environmental variability, less time consuming, and suitable for long juvenile plants such as citrus (De Mori & Cipriani, 2023). There are several resistance and susceptible genes related to HLB disease which could be the targets in molecular marker development for citrus resistance evaluation to HLB, such as *WRKY70* (Mafra et al., 2012), *callose synthase 7* (Granato et al., 2019), *Accelerated Cell Death 2* (*ACD2*) (Pang et al., 2020), *NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1* (*NPR1*) (Wu et al., 2021), *salicylic acid binding protein 2* (*SABP2*) (Dong et al., 2024), and *calcium-dependent protein kinase 6* (*CPK6*) (Zhang et al., 2024). Previous studies from Nugroho et al. (2025a; 2025b) identified a single nucleotide polymorphism (SNP) in *callose synthase 7* and *WRKY70* gene fragment sequences, respectively, that could discriminate between resistant/tolerant and susceptible citrus genotypes.

The focuses of this study were to perform *in silico* analysis of the nucleotide variations in *NPR1* gene sequence among several citrus genotypes whose resistance information to HLB have been known previously and employed phylogenetic analysis among them. The information obtained in this study, especially the nucleotide variations among the citrus genotypes, is expected to be promising tool for designing new functional markers that is suitable for fast and reliable screening of citrus genotypes based on HLB resistance.

Materials and Methods

Collecting of Citrus NPR1 Gene Sequence

Initially, the *NPR1* gene sequence was obtained from the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/). The sequence was then used as the input in the Basic Local Alignment Search Tool nucleotide (BLASTn) program (Altschul et al., 1997) in the Citrus Genome Database (https://www.citrusgenomedb.org/blast/nucleotide/nucleotide), compared to *Citrus sinensis* v1.0 genome (JGI) coding sequence (CDS). The sequence collected from BLASTn result was subsequently used as the reference sequence to collect the *NPR1* gene sequences from 20 genotypes

which consisted of 14 citrus and six relative's genotypes analyzed in this study. All the 20 genotypes have been known for their resistance from several references as presented in Table 1.

Data Analysis

The *NPR1* gene sequences, which were collected *in silico* via Citrus Genome Database, were further analyzed using multiple sequence alignment program in Clustal Omega (https://www.ebi.ac.uk/jdispatcher/msa/clustalo) (Sievers & Higgins, 2014) and Geneious 10.0.3 trial version (Kearse et al., 2012) to identify the nucleotide variations, including single nucleotide polymorphisms (SNPs), insertions, or deletions. The phylogenetic analysis was employed to the *NPR1* gene sequences using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) program and the Tamura-Nei model, with 1,000 bootstrap replications in Mega X (Kumar et al., 2018).

Results and Discussion

The investigation of *NPR1* gene sequence in the NCBI database successfully identified an accession number of NM_105102.3 that described the *Arabidopsis thaliana* regulatory protein (NPR1) mRNA. This sequence displayed the similarity ranging from 70.92 to 79.43% to the orange1.1g007923m locus of *C. sinensis* v1.0 genome (JGI) CDS (Figure 1), with *E*-value of 4.52166E-36. The gene located in orange1.1g007923m locus showed annotation as protein binding according to AmiGO 2 (https://amigo.geneontology.org/amigo/term/GO:0005515) and regulatory protein NPR1 according to KEGG Orthology (https://www.genome.jp/dbget-bin/www_bget?ko:K14508). The gene sequence possessed a total length of 3,785 bp, with the coding sequence (CDS) length of 1,754 bp.

The CDS possessed four intermittent exons with three introns and one untranslated region (UTR) at the 5'-end, as presented in Figure 2. The gene

Table 1. List of 20 genotypes used in this study.

No.	Genotype name	Resistance information	References
1	*Citrus sinensis* cv. Jinhong	Susceptible	Ramadugu et al. (2016)
2	*Citrus sinensis* cv. Neixiu	Susceptible	Ramadugu et al. (2016)
3	*Citrus clementina*	Susceptible	Ramadugu et al. (2016)
4	*Citrus reticulata* cv. Ponkan	Susceptible	Ramadugu et al. (2016)
5	*Citrus reticulata* cv. Murcott	Susceptible	Ramadugu et al. (2016)
6	*Citrus limon* Eureka	Susceptible	Ramadugu et al. (2016)
7	*Citrus maxima* Cupi Majiayou	Susceptible	Ramadugu et al. (2016)
8	*Citrus maxima* cv. Huazhouyou-tomentosa	Susceptible	Ramadugu et al. (2016)
9	*Citrus ichangensis* cv. ZGYCC	Tolerant	Wu et al. (2020)
10	*Citrus glauca* CRC3463	Resistant	Ramadugu et al. (2016)
11	*Citrus inodora* CRC3784	Tolerant	Ramadugu et al. (2016)
12	*Citrus mangshanensis* cv. MSYG	Tolerant	Zhao et al. (2017)
13	*Citrus australis*	Tolerant	Ramekar et al. (2025)
14	*Citrus australasica* cv. Rainbow	Tolerant	Weber et al. (2022)
15	*Clausena lansium* cv. HP	Susceptible	Ding et al. (2005)
16	*Atalantia buxifolia* cv. HKC	Tolerant	Hijaz et al. (2016)
17	*Citropsis gilletiana* cv. CGI	Resistant	Alves et al. (2021)
18	*Fortunella hindsii* S3y-45	Unknown	Ramadugu et al. (2016)
19	*Poncirus trifoliata* DPI 50-7	Tolerant	Hall et al. (2017)
20	*Murraya paniculata* cv. Kunming	Resistant	Ramadugu et al. (2016)

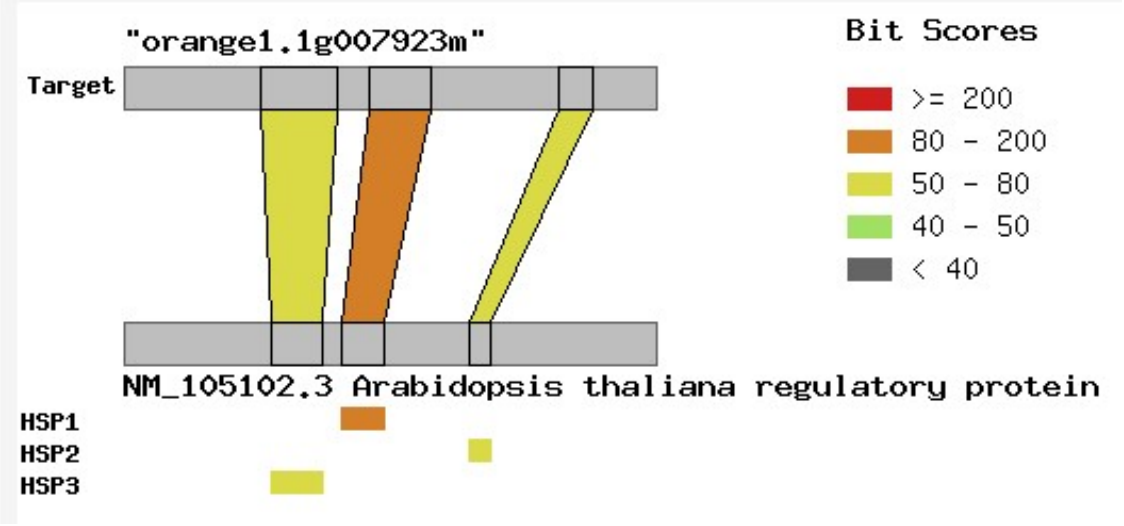

Fig. 1. The BLASTn result of *NPR1* gene sequence from NCBI with *Citrus sinensis* v1.0 genome (JGI) CDS data in Citrus Genome Database.

sequence from orange1.1g007923m locus was subsequently utilized as the reference sequence to collect the *NPR1* gene sequences from 20 genotypes used in this study. The reference sequence was compared to the citrus genome data available in Citrus Genome Database, followed by analysis using multiple sequence alignment program. The homology analysis outcome is presented in Table 2. The homology analysis showed similarity which ranged from 92.7 to 100%, between the reference sequence with the *NPR1* gene sequences from 20 genotypes, with *E*-value of 0.

NPR1 is one of the resistance genes in citrus defend systems against HLB disease. This gene plays a crucial role as a regulator in the activation of systemic acquired resistance (SAR) pathway (Wu et al., 2021). The *NPR1* gene induces the activity of *WRKY70* gene to activate the salicylic acid pathway and repress the jasmonic acid pathway (Qiu et al., 2020). Previously, Li et al. (2004) reported that *NPR1* gene was working on the upstream position of *WRKY70* gene in the salicylic acid-dependent pathway. On the other hand, Dutt et al. (2015) reported the resistance improvement of transgenic *C. sinensis* Hamlin and Valencia cultivars which were introgressed by *NPR1* gene from *A. thaliana* to HLB disease.

In this study, we identified nucleotide variations in *NPR1* gene sequences which consisted of SNPs, insertions, and deletions. Interestingly, there were six unique SNPs that could distinguish between susceptible and resistant citrus genotypes as displayed in Table 3. The SNPs were located at the position of 52, 88, 173, 184, 330, and 3,383 bp downstream from START codon of the reference sequence. Unlike the previous studies from Nugroho et al. (2025a; 2025b) which identified a notable SNP at the intron region, all the interesting SNPs in this study were located in exon

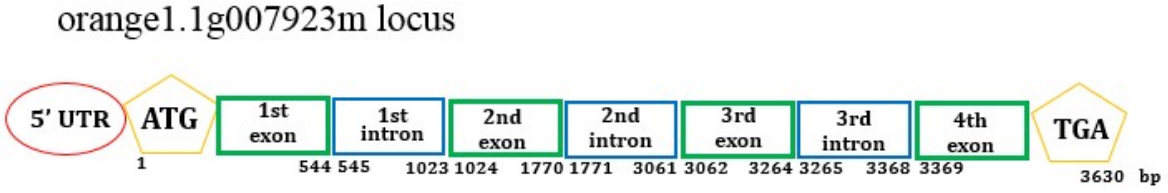

Fig. 2. The coding sequence structure of *NPR1* gene located at orange1.1g007923m locus.

Table 2. The homology analysis results between *NPR1* reference sequence with citrus genome data available at Citrus Genome Database.

No.	Reference locus	Genome target	Homolog locus	Similarity (%)	*E*-value
1	orange1.1g007923m (89173..92957+)	*Citrus sinensis* cv. Jinhong v1.0 genome	drCitSine.Jinhong.1.0.HB.chr4 (12586751... 12582944)	99.97	0
2		*C. sinensis* cv. Neixiu v1.0 genome	Chr04 (11184261...11180456)	99.89	0
3		*C. clementina* v1.0 genome (JGI) CDS	Ciclev10007854m (10530304..10534418-)	100	0
4		*C. reticulata* cv. Ponkan v1.0 genome	chr4 (19954948...19958755)	99.97	0
5		*C. reticulata* cv. Murcott v1.0 genome	C.murc_hap1_04_h1 (11063158... 11059351)	99.97	0
6		*C. limon* Eureka v1.0 genome	GWHCBFQ00000007.1 (10988089...10984282)	100	0
7		*C. maxima* Cupi Majiayou v1.0 genome	chr4 (26262200... 26265092)	98.83	0
8		*C. maxima* cv. Huazhouyou-tomentosa (HZY-T) v1.0 genome	chr4 (22557936... 22560838)	98.94	0
9		*C. ichangensis* cv. ZGYCC v2.0 genome	contig148 (5336503.. 5340351)	98.95	0
10		*C. glauca* CRC3463 v1.0 genome	Pri_Scaffold_1 (15497505.. 15500425)	98.52	0
11		*C. inodora* CRC3784 v1.0 genome	Alt_Scaffold_1 (11067825...11063985)	98.95	0
12		*C. mangshanensis* cv. MSYG v1.0 genome	Contig181 (731354..727570)	99.04	0
13		*C. australis* v1.0 genome, scaffolds	Chr4 (18921993... 18919096)	98.93	0
14		*C. australasica* cv. Rainbow v1.0 genome	Chr04.H2 (10749350... 10750322)	99.26	0
15		*Clausena lansium* cv. HP v1.0 genome	contig84 (10041303... 10037493)	92.7	0
16		*Atalantia buxifolia* cv. HKC_v2.0 genome	chr4 (8450634... 8454477)	96.94	0
17		*Citropsis gilletiana* cv. CGI v1.0 genome	ctg000216 (12027972... 12031706)	96.54	0
18		*Fortunella hindsii* S3y-45 v1.0 genome CDS	sjg283810.4 (1909857..1914501+)	99.74	0
19		*Poncirus trifoliata* DPI 50-7 v1.3.1 genome CDS	Ptrif.0001s0987.3.v1.3.1 (10079790..10084055-)	99.34	0
20		*Murraya paniculata* cv. Kunming v1.0 genome	Chr06 (14652916..14656679	93.25	0

areas. As many as five SNPs were non-synonymous and promoted amino acid changes while a SNP at the position of 330 bp downstream from START codon was synonymous and did not cause amino acid change. However, the presence of synonymous SNP allegedly could promote several effects to DNA, RNA, and splicing properties (Zeng & Bromberg, 2019). First, the presence of synonymous SNPs in the codons related to transcription factors (TF) can affect the binding ability of TF and gene transcription rates, which caused the change of methylation patterns by disturbing the site-specific of GC compositions. The existence of synonymous SNPs could also affect the codon bias and mRNA stability which

Table 3. List of interesting SNPs identified in *NPR1* gene sequences in this study.

SNP position downstream from START codon in reference sequence (bp)	52	88	173	184	330	3,383
NPR-1 reference sequence (*Citrus sinensis*)	C	T	A	T	A	T
C. sinensis cv. Jinhong	C	T	A	T	A	T
C. sinensis cv. Neixiu	C	T	A	T	A	T
C. clementina	C	T	A	T	A	T
C. reticulata cv. Ponkan	C	T	A	T	A	T
C. reticulata cv. Murcott	C	T	A	T	A	T
C. limon Eureka	C	T	A	T	A	T
C. maxima Cupi Majiayou	A	A	C	A	G	C
C. maxima cv. Huazhouyou-tomentosa	A	A	C	A	G	C
C. ichangensis cv. ZGYCC	A	A	C	A	G	C
C. glauca CRC3463	A	A	C	A	G	C
C. inodora CRC3784	A	A	C	A	G	C
C. mangshanensis cv. MSYG	A	A	C	A	G	C
C. australis	A	A	C	A	G	C
C. australasica cv. Rainbow	A	A	C	A	G	C
Clausena lansium cv. HP	A	A	C	A	G	C
Atalantia buxifolia cv. HKC	A	A	C	A	G	C
Citropsis gilletiana cv. CGI	A	A	C	A	G	C
Fortunella hindsii S3y-45	A	A	C	A	G	C
Poncirus trifoliata DPI 50-7	A	A	C	A	G	C
Murraya paniculata cv. Kunming	A	A	C	A	G	C
SNPs position region	First exon	First exon	First exon	First exon	First exon	Fourth exon
SNPs type*	NS	NS	NS	NS	S	NS
Amino acid change	Arginine to serine	Serine to threonine	Glutamine to proline	Tyrosine to asparagine	-	Valine to alanine

The red letters represented SNPs that were possessed by susceptible genotypes and the black letters represented SNPs that were were possessed by resistant/tolerant genotypes.
*NS = non-synonymous, S = synonymous

might correlate with the level of expression, translation rate, and protein structure.

Additionally, the synonymous SNPs could also affect the splicing process by changing the affinity of pre-mRNA to spliceosome, which might result in incorrect identification of exon and intron regions, leading to production of abnormal mRNA and non-functional protein. In this study, we did not identify insertions or deletions with unique patterns which could distinguish between susceptible and resistant citrus genotypes.

In this study, we conducted a fully *in silico* study to collect the *NPR1* gene sequences, without performing molecular analysis as in previous studies (Nugroho et al., 2025a; 2025b). Another differences are the absence of Carrizo citrange (*C. sinensis* × *Poncirus trifoliata*) and rough lemon (*Citrus jambhiri*) genotypes, which were used in our previous studies, due to the unavailability of their genome data in the Citrus Genome Database, and the elimination of several citrus genotypes without HLB resistance information such as *Citrus linwuensis*, *C. changshanensis*, *Citrus garrawayi*, and *Citrus hongheensis*. According to SNP pattern, all of the susceptible citrus genotypes such as *C. sinensis*, *Citrus clementina*, *Citrus reticulata*, and *Citrus limon* possessed similar alleles. The phylogenetic tree also revealed it, where they tended to group together in similar cluster (Figure 3). However, the *Citrus maxima* genotypes, both Cupi Majiayou and Huazhouyou-tomentosa cultivars, and *Clausena lansium*

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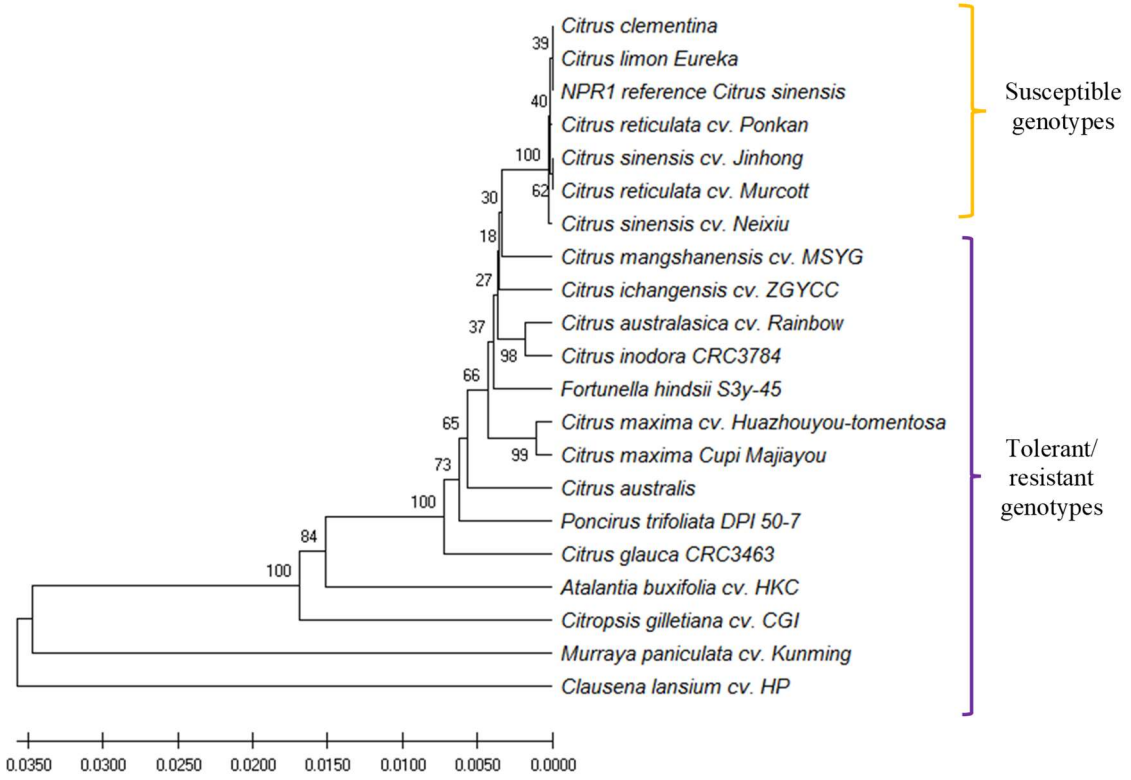

Fig. 3. The phylogenetic tree constructed from *NPR1* gene sequences of 20 genotypes used in this study, based on UPGMA method and Tamura-Nei model.

consistently possessed similar alleles with the tolerant/resistant genotypes (Table 3). The phylogenetic tree also showed their clustering pattern together with the resistant genotypes (Figure 3).

All commercial citrus cultivars such as sweet orange (*C. sinensis*) and mandarin (*C. reticulata*) are well known as susceptible to HLB disease (Bové, 2006; Pandey et al., 2022; Tsai et al., 2008). Likewise, *C. clementina* and *C. limon* are also known as susceptible genotypes (Ramadugu et al., 2016). Meanwhile, pomelo (*C. maxima*) was reported to be susceptible to HLB disease in several studies (Puttamuk et al., 2014; Ramadugu et al., 2016; Yu et al., 2022). A study from Widyaningsih et al. (2019) also reported the susceptibility of *C. maxima* cv. Nambangan to HLB infections. On the contrary, previous study from Prasetyaningrum et al. (2012) revealed the resistance of several pomelo cultivars such as Pangkajene Putih, Magetan, Raja, and Pangkajene Merah to HLB infection. Our previous studies (Nugroho et al., 2025a; 2025b) using *callose synthase 7* and *WRKY70* gene-specific primers also revealed the existence of the alleles in *C. maxima* cv. Pangkajene Putih and Magetan which were similar to the resistant/tolerant genotypes. According to Tsai et al. (2008), before 1970s, pomelo was tolerant to HLB disease. However, after that period, pomelo became susceptible due to the appearance of new bacterial strain. A study from Cao et al. (2015) revealed the tolerance of pomelo to HLB infection. This was proved by the nitrogen:phospor (N:P) ratio of live leaf and fruit yield which were unaffected by HLB infection compared to *C. reticulata*, since the HLB infection will affect the P resorption in infected plants.

Chinese wampee (*C. lansium*) was previously reported to be infected by HLB disease but with low titer of bacteria (Deng et al., 2007; Ding et al., 2005; Lin et al., 2010). Ramadugu et al. (2016) did not use

C. lansium in their study but reported the resistance of pink wampee (*Clausena excavata*) to the HLB strain from Florida. However, our study demonstrated that *C. lansium* consistently possessed similar alleles with the resistant genotypes. Transcriptomic study performed by Liao et al. (2025) also revealed the different gene expressions between HLB-infected resistant *C. lansium* and susceptible *C. reticulata* cv. Ponkan. While Ponkan mandarin mainly defended against HLB infection via lignin synthesis and cell wall modification pathways, *C. lansium* defended against HLB using cellular homeostasis and metabolism regulation.

Another genotype of Hong Kong kumquat (*Fortunella hindsii*), according to Ramadugu et al. (2016) study, was uncategorized for HLB resistance due to the insufficient data. Previous study from Tsai et al. (2006) reported the susceptibility of *Fortunella margarita* to HLB infection in Taiwan. Shokrollah et al. (2009) categorized *Fortunella* sp. cv. Kasturi Chinai as moderate with severity disease ranging from 41–50%. A study from Folimonova et al. (2009) demonstrated the chlorotic leaves and plant growth reduction in HLB-infected Meiwa kumquat (*Fortunella crassifolia*). However, *Fortunella* spp. is classified as the poor host for HLB vector (*D. citri*) (Hall et al., 2012).

Our study results also demonstrated that *F. hindsii* also possessed similar alleles with the resistant genotypes and clustered together in phylogenetic tree, as well as *C. maxima* and *C. lansium*. Our previous study (Nugroho et al., 2025b) also showed the existence of the allele in *F. hindsii* which was similar to resistant/tolerant genotype based on *WRKY70* fragment gene sequence. The rest of the genotypes such as *Citrus mangshanensis*, *Citrus ichangensis*, *Citrus glauca*, *Citrus inodora*, *Citrus australis*, *Citrus australasica*, *P. trifoliata*, *Citropsis gilletiana*, *Atalantia buxfolia*, and *Murraya paniculata* showed no contradictions in either their alleles or clustering pattern in phylogenetic tree, since they have been clearly categorized as tolerant/resistant genotypes in several studies (Alves et al., 2021; Hall et al., 2017; Hijaz et al., 2016; Ramadugu et al., 2016; Ramekar et al., 2025; Weber et al., 2022; Wu et al., 2020; Zhao et al., 2017).

Marker-assisted selection (MAS) is necessary in citrus breeding program due to the long juvenile period of these plants, the apomixis and polyembryony phenomena, as well as high heterozigosity. These made the citrus breeding programs more challenging (Muñoz-Fambuena et al., 2019; Xu et al., 2022). The discovery of unique SNPs that could distinguish between resistant and susceptible citrus genotypes in this study is very useful for designing new functional markers such as single nucleotide amplified polymorphism (SNAP), a functional marker developed based on reference (Ref) or alternate (Alt) alleles from the SNP that existed in certain positions of the plant genome. The genotype which possesses the reference allele will produce an amplicon band by using the SNAP marker that was designed based on the reference allele, while the genotype which possesses the alternate allele will not show the amplicon band. The use of SNAP marker for selection tool in plant breeding activities is promising and have previously been reported for several agricultural commodities in Indonesia such as chili pepper (Terryana et al., 2020), cacao (Tarigan et al., 2021), *Phalaenopsis* (Sukma et al., 2021), coconut (Pesik et al., 2017), papaya (Noflindawati et al., 2021), and salacca (Prihatini et al., 2022).

In addition, the SNAP markers might be used as the selectable marker especially to help the artificial inoculation method of HLB disease which is still challenging due to the complexity of bacterial culture issue. The presence of selection tool for fast and reliable screening of citrus genotypes based on HLB resistance in future studies is expected to accelerate the citrus breeding program in Indonesia to reduce HLB disease.

Conclusion

Nucleotide variations analysis of *NPR1* gene sequences in 20 genotypes which consisted of 14 citrus and six relative's genotypes via *in silico* study identified six interesting SNPs that could distinguish between susceptible and resistant citrus genotypes. The SNPs were located at the position of 52, 88, 173, 184, 330, and 3,383 bp downstream from START codon in the reference sequence. A total of five SNPs were non-synonymous and promoted amino acid changes while one SNP at the position of 330 bp downstream from START codon was synonymous and did not cause amino acid change. The phylogenetic analysis also revealed the separation between susceptible and resistant citrus genotypes in two main clusters. The SNPs finding in this study is expected to be useful for designing new functional marker such as SNAP that can be used as screening tools of citrus genotypes based on HLB resistance in future studies.

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Conflict of Interest

All authors have no conflicts of interest to disclose.

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