Plant defense-related gene expression analysis of canker-infected lime seedling

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Abstract. A serious problem for Paan-lime production in Thailand is a citrus canker disease caused by the bacterium Xanthomonas citri subsp. citri (Xcc). The use of antagonist against pathogenic Xcc and a bacterial antagonist on defense-related gene expression of Paan-lime. The ability of Xcc and the antagonist Pseudomonas aeruginosa SWUC02 to induce defense-related gene expression, including PR-1, Pt14, LRR8, and LOX gene, was assessed. We assured that all four defense-related genes are present in Paan-lime by analyzing the similarity of nucleotide sequences in parts of the genes against other citrus species. Using reverse-transcription PCR (RT-PCR), we showed that the antagonist is able to induce the expression of LRR8 gene at 24 hours post-inoculation, while Xcc induces PR-1 and LOX gene expression at the same time. Our results suggest that these defense-related genes alter their expressions in response to canker disease infection. Thus, we could use this group of genes as a biomarker for screening canker-resistance Paan-lime tree.

1. Introduction
Canker disease caused by Xanthomonas citri subsp. citri (Xcc) is a major problem for citrus fruit family production worldwide [1]. In Thailand, there is a variety of citrus fruit species that are susceptible to Xcc, especially the Paan-lime (Citrus aurantifolia) [2]. The common way to limit canker disease in Paan-lime is to use chemicals copper oxychloride or copper hydroxide. Alternatively, to avoid unknown chemical effects, the use of biological agents to control the disease has become a preferred practice [3]. In addition, disease resistance cultivars were isolated [4]. To date, our understanding of gene regulations involved in plant defense response has been limited; this presents a key challenge for selecting and breeding strategies to obtain novel resistant cultivars. To the best of our knowledge, the Paan-lime defense-related gene identities and their expressions have not been examined.

Plants are equipped with various strategies to fight against invading phytopathogens, including an increase in cell wall strength, a production of certain metabolites to kill pathogens, and a modulation of defense-related gene expressions in plant cells [5]. In plant defense system, pattern recognition receptor (PRR) located in transmembrane detects a pathogen-associated molecular pattern (PAMP) such as pathogen proteins [6]. Successful recognition between PRR and PAMP leads to PAMP-
triggered immunity (PTI) [6]. Some virulent pathogens, however, utilize effector proteins to suppress PTI-associated responses – this phenomenon is known as effector-triggered susceptibility (ETS) [6]. To overcome ETS, plant cells use the nucleotide-binding site-leucine-rich repeat (NBS-LRR) proteins to recognize pathogen effector proteins [6]. The NBS-LRR proteins are typically encoded by the resistance genes (R genes) [6]. The successful recognition between NBS-LRR and pathogen effector results in effector-triggered immunity (ETI) [6]. Both PTI and ETI induce various plant defense mechanisms, such as systemic acquired resistance (SAR), increased hypersensitive response (HR), and a production of phytoalexin to inhibit pathogen growth and its activities [5].

Several genes are involved in PTI and ETI; however, the scope of this study is limited to four representative genes, including putative citrus canker resistance (Pt14) gene, leucine-rich repeat receptor-like kinase (LRR8) gene, pathogenesis-related 1 (PR-1) gene, and lipoxygenase (LOX) gene. Pt14 gene is one of the Xcc resistance genes found in an intergenic hybrid of Poncirus trifoliata and Citrus grandis, whose protein belongs to the Toll/Interleukin-1 receptor homology (TIR) group of NBS-LRR domains involved in ETI [7]. The combination of Pt14 with the restriction enzyme Bfa1 has been reported to be an effective marker gene for screening canker-resistant lime cultivars [8]. Leucine-rich repeat receptor-like kinase (LRR-RLK) plays an important role in abiotic and biotic stresses, including recognition of PAMP and induction of PTI [9]. Pathogenesis-related (PR) gene expression has been used for monitoring plant defense in response to pathogens in several studies [10, 11]. PR genes are typically divided into 17 groups involved in producing a variety of enzymes and antibiotics to suppress phytopathogens [12]. LOX gene has diverse functions in higher plants, including the plant defense response against phytopathogen attack. Products of LOX gene family include antimicrobial metabolites and volatiles; they are involved in jasmonic acid production, a phytohormone required for the induction of systemic resistance (ISR) against pathogens [13].

In addition to phytopathogen-incuced plant immunity, some antagonists are able to simultaneously promote ISR and inhibit phytopathogen growth. For example, Bacillus cereus AR156 triggers the expression of PR genes in Arabidopsis thaliana, such as PR-1, PR-2, and PR-5, to produce PR proteins against Pseudomonas syringae pv. tomato [11]. Saravanakumar et al. al. reported that Pseudomonas fluorescens Pf1 has the potential to reduce blister blight disease caused by Exobasidium vexans in Camellia sinensis, and to induce the plant defense enzymes, such as peroxidase, phenylalanine ammonia-lyase, and chitinase [14]. Here, we use X. citri subsp. citri (Xcc) and Pseudomonas aeruginosa SWUCO2 as a representative of phytopathogen and an antagonist of the lime seedling, respectively. We aim at identifying the defense-related genes in Paan-lime and determining their expressions during infection and biocontrol process, in which the plant is concomitantly inoculated with Xcc and the antagonist SWUCO2.

2. Materials and methods

2.1. Defense-related gene analysis

For genomic DNA (gDNA) extraction from Paan-lime, leaves were ground in liquid nitrogen, and gDNA was extracted by AccuPrep® Genomic DNA extraction kit. Quality and quantity of the gDNA was examined with agarose gel electrophoresis and spectrophotometry, respectively. The existence of defense-related genes, PR-1, Pt14, LRR8, LOX, and a housekeeping gene, Ef-1a, in gDNA of Paan-lime was analyzed by PCR with modified primer pairs previously designed from A. thaliana [10], C. grandis x P trifoliata [7], Citrus clementina [10], C. sinensis [15], and C. clementina [15], respectively. Primer pairs containing HindIII and EcoRI restriction sites (Table 1) were used to identify defense-related genes. KOD-Plus-Neo DNA polymerase was used for PCR reaction with the following conditions: 95°C for 2 min, 30 cycles of 98°C (10 sec), 55-61°C (30 sec), 68°C (30 sec), and finally 68°C for 7 min. PCR products were purified with HiYieldTM Gel/PCR DNA fragments extraction kit and digested with HindIII and EcoRI. The resulting DNA fragments were ligated with pBluescript SK (+) which was cut with the same restriction enzymes. The ligated DNA was transformed into Escherichia coli JM109. Transformants were screened by blue-white screening, and plasmids were screened by restriction mapping with BamHI and XhoI. The recombinant plasmids that contain PR-1, Pt14, LRR8, LOX, or EF-1a gene were sent to FASMAC co. (Japan) for sequencing with M13M3 primer (5′
GTAAAACGACGGCCAGT 3') and M13RV primer (5' CAGGAAACAGCTATGAC 3'). The nucleotide sequences of the defense-related genes were analyzed by Blastn against GenBank database, and pairwise alignment was performed with EMBOSS MATCHER (European Bioinformatics Institute) program.

### Table 1. Modified primer sets for use in this study.

| Primer names | Primer sequences (5´-3´)1, 2 | Specific regions | Tm (°C) |
|--------------|-------------------------------|------------------|---------|
| rePR1f       | f- CCCTAAGCTTACAAACACACATCTCCGAAA | Pathogenesis-related (PR-1) gene | 60.2    |
| rePR1r       | r- GCATGAATTCTTGAAATGAGCAGCAGCAAA |                             | 58.8    |
| rePt14up     | f- ATTTAAGCTTGACATATCTCTTATCAGTT | Putative citrus canker resistance (Pt14) gene | 55.3    |
| rePt14lw     | r- TACTGAATTCCGAGCTCAAAAATAACCATCTG |                             | 57.4    |
| reLRR8f      | f- ACGGAAAGCTTTGCACCAAGAGCTAGCTACA | Leucine-rich repeat receptor kinase (LRR8) gene | 64.3    |
| reLRR8r      | r- GACGAAAATTCGAGTAGATGGAAGCCCGAAG |                             | 61.5    |
| reLOXf       | f- TGCCAAAGCTTGTGTTTCTGGAACCTTGCTG | Lipoxygenase (LOX) gene | 64.3    |
| reLOXr       | r- TTATGAAATTCCTGTGATTGCACAGGCGTCC |                             | 61.5    |
| reEf-1af     | f- TTTAAGCTTACATGATTACCGGTACCTCA | Elongation factor 1-alpha (EF-1α) gene | 57.4    |
| reEf-1ar     | r- CATCGAATTCCACCAAGGGGTGAAAGCAAG |                             | 61.5    |

1 Bold and underlined letters refer to restriction site of HindIII and EcoRI, respectively.
2 f and r denote forward and reverse primers, respectively.

#### 2.2. Plant inoculation and total RNA extraction

We used Paa n-lime seedlings grown in tissue culture conditions to eliminate other external factors while preserving sufficient nutrition. *P. aeruginosa* SWUC02 was chosen as an antagonist because it could inhibit Xcc on Pa an-lime seedling without posing any negative effects on the seedling in the tissue culture condition (unpublished data). The Pa an-lime seedlings were divided into four groups for inoculation: (1) Xcc, (2) *P. aeruginosa* SWUC02, (3) Xcc + *P. aeruginosa* SWUC02, and (4) uninoculated (mock). The seeds were surface sterilized with 0.25% NaOCl and washed with sterile water. Seeds were placed on Murashige and Skoog (MS) medium in tissue culture conditions. Seedlings with 4–7 mature leaves were used, and leaves were punched by a sterile needle.

The first inoculation was performed with *P. aeruginosa* SWUC02 at time 0, and the second inoculation was done with Xcc after 24 hours of incubation. The *P. aeruginosa* SWUC02 at 10⁶ CFU/mL and Xcc at 10⁶ CFU/mL were used to swab the punched leaves. The negative control was swabbed with sterile TSB twice, and the positive control was swabbed with sterile TSB and then inoculating with Xcc.

After 24 and 48 hours post-inoculation (hpi) of Xcc, the leaves of seedlings were ground in liquid nitrogen. Total RNA from the seedling leaves was extracted. Genomic DNA in the total RNA sample was eliminated by DNase I. The quantity and quality of total RNA were examined by spectrophotometry and agarose gel electrophoresis, respectively.

#### 2.3. Reverse transcription PCR

Reverse transcription PCR (RT-PCR) was performed under the following conditions: 45 °C for 20 min, 95 °C for 1 min, 30 cycles of 95 °C for 10 sec, 50 °C for 10 sec, and 72 °C for 35 sec, and finally one cycle of 72 °C for 5 min. Total RNA was used as a template, and the primer pairs specific to the PR-1, Pr14, LRR8, LOX gene (defense-related gene), or EF-1α gene (a housekeeping gene as a control) were used. The PCR products were examined by agarose (1.5%) gel electrophoresis stained with ethidium bromide. Image Lab 6.0 software (Bio-Rad Laboratories, Inc., USA) was used to convert DNA band intensities to pixel values (grayscale). Relative gene expression was calculated by the ratio of the defense-related gene band intensity value over the EF-1α band intensity value. This experiment
was performed with three biological replicates. Statistical tests were performed over the relative gene expression percentage by ANOVA and Tukey’s HSD (P < 0.05) for multiple comparisons.

3. Results

3.1. Analysis of defense-related gene of Paan-lime

We use primer pairs originally used in other species (Table 1) to detect PR-1, Pt14, LOX, LRR8 and EF-1a genes in Paan-lime by PCR. The sizes of PCR products were smaller than 100, 450, 350, 200, 150 bp, respectively (Figure 1). For similarity comparison of a part of these genes between Paan-lime and other citrus species, we found that PR-1, Pt14, LOX, LRR8, and EF-1a were 100%, 100%, 62%, 97%, and 100% similar to C. sinensis (accession No. XM_025097265.1), C. aurantifolia (accession No. HQ875732.1), C. sinensis (accession No. XM_006475281.3), C. sinensis (accession No. XM_006465842.3), and C. clementina (accession No. XM_006424569.2), respectively. For pairwise alignment results, the amplicon sizes of PR-1, Pt14, LRR8, and EF-1a of Paan-lime were similar to their counterparts in other citrus cultivars. However, LOX PCR product size in Paan-lime (331 bp) differs from C. sinensis (175 bp) (Table 2).

![Figure 1](image_url)

Figure 1. Identification of defense-related genes and a house keeping gene in Paan-lime: A; PR-1 (<100 bp), B; Pt14 (450 bp), C; LOX (350 bp), D; LRR8 (200 bp) and Ef-1a (150 bp), Lane M; 100 bp DNA ladder.

| Genes | Amplicon size (bp) | Citrus species | Specific regions | % Similarity |
|-------|-------------------|----------------|------------------|-------------|
| **PR-1** | 71 | **C. sinensis** | Pathogenesis-related protein PR-1 gene | 100 |
| **Pt14** | 426 | **C. aurantifolia** | Putative citrus canker resistance protein Pt14R gene | 100 |
| **LRR8** | 177 | **C. sinensis** | MDIS1-interacting receptor like kinase 2-like | 97 |
| **LOX** | 331 | **C. sinensis** | Linoleate 13S-lipoxygenase 3-1 | 62 |
| **Ef-1a** | 133 | **C. clementina** | Elongation factor 1-alpha | 100 |

1 Sequences were obtained from the NCBI GenBank database.
Figure 2. RT-PCR analysis of defense-related genes in Paan-lime: this figure is divided into two panels, electrophoresis analysis of cDNA (left) and percentage of relative gene expression (right). A and B; PR-1, C and D; Pt14, E and F; LRR8, and G and F; LOX. hpi = hours post-inoculation. Neg means a negative control that used nuclease-free water instead of RNA template. Error bars represent standard deviation. Different letters (a, b, A, B, C) above error bars indicate statistical differences by Tukey’s HSD test (P < 0.05).

3.2. Defense-related gene expression of infected Paan-lime
We determined gene expression of PR-1, Pt14, LOX, and LRR8 by reverse transcription PCR when it was triggered by phytopathogen or antagonist. The expression of PR-1 gene was induced by pathogenic Xcc at 24 hpi and was slightly decreased at 48 hpi while the same expression was not
induced by the antagonist, *P. aeruginosa* SWUC02 (Figure 2A and 2B). A similar pattern was found for LOX gene expression (Figure 2G and 2H). In addition, the *Xcc* infected seedling showed higher LOX gene expression than that of uninoculated seedling, and the expression decreased at 48 hpi (Figure 2G and 2H). *P. aeruginosa* SWUC02 could only induce the LRR8 expression at 24 hpi (Figure 2E and 2F). Interestingly, neither *Xcc* nor *P. aeruginosa* SWUC02 could induce the expression of *Pt14* gene at 24 or 48 hpi (Figure 2C and 2D).

4. Discussion

Among the selected defense-related genes in our study, *PR-1* and *Pt14* gene have 100% similarity to those from *C. sinensis* and *C. aurantifolia*. Our gene expression analysis during *Xcc* infection supports the use of *PR* gene as a biomarker for detecting defense-related gene expression. Although we have not analyzed PR protein activities, we think PR-1 protein from Paan-lime could function in plant defense response, since PR proteins from different citrus species were shown to perform similar functions in plant defense against phytopathogens [12,16]. In addition, LRR-RLK gene was used for evolution study in the citrus plant [17]. Our results showed that LRR8 of Paan-lime (*C. aurantifolia*) is similar to *C. sinensis* with 97% identity. However, LRR8 sequence in *C. sinensis* is different from that presented in *C. clementina* [17]. Interestingly, LOX gene of Paan-lime has only 62% similarity to that from *C. sinensis*. The relative low similarity could be due to the action of a transposable element, which was previously reported in legume [18] and maize [11].

Our RT-PCR analysis showed that *PR-1* and LOX gene expressions were increased in response to pathogenic *Xcc* at 24 hpi. Our result differs from a study by Waewthongrak et al., showing that the expression of LOX gene in *C. sinensis* was stimulated at 48 and 72 hours after pathogen infection, and lipoxygenase activity was concurrently detected [15]. However, pathogenic *Xcc* and the antagonist *P. aeruginosa* SWUC02 did not induce the expression of *Pt14* gene in Paan-lime at 24 and 48 hpi. The failure of inducing *Pt14* gene expression might be attributed to unsuitable period of inoculation time. In addition, the expression of LRR8 gene was triggered by the antagonist *P. aeruginosa* SWUC02 at 24 hpi. Our result supports an idea that a competent antagonist is capable of inducing systemic resistance in plants. For example, if tomato plants are pretreated with the antagonist *P. putida* LSW17S prior to pathogen attack, they will become more resistant to pathogens due to H2O2 production and the induction of *PR* genes expression [19]. Another study reported that the use of synthetic bacterial volatile compound, which mimics the antagonist compound, could induce the expression of LRR-transmembrane kinase and NBS-LRR gene. The expression of these genes induces the resistance of *Agrostis stolonifera* to *Rhizoctonia solani* [20]. A few other studies reveal the ability of *P. fluorescens* to enhance defense-related gene expression [21, 22]. Together, our results and others’ independent observations suggest that different defense-related genes are expressed in a time-dependent and microbe-specific manner.

In summary, defense-related genes expression in Paan-lime is an important mechanism responding to canker disease infection. We found that *PR-1* and LOX gene expressions were induced by bacterial *Xcc* pathogen, while LRR8 was induced by antagonist *P. aeruginosa* SWUC02, suggesting that the inductions are microbe-specific. We suggest the use of *PR-1* and LOX gene expression as a biomarker for screening canker-resistant Paan-lime trees.

5. References

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