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Molecular characterization of *PepSTC* and *PepCYP* involved in the biosynthesis of a novel phytoalexin in the transgenic *N. benthamiana*

조선대학교 대학원

글로벌바이오융합학과

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형질전환된 담배에서 신규의 phytoalexin 합성에 관여하는
*PepSTC*와 *PepCYP* 유전자의 분자적 특성 규명

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ABSTRACT

Molecular characterization of *PepSTC* and *PepCYP* involved in the biosynthesis of a novel phytoalexin in the transgenic *N. benthamiana*

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Plant anthracnose (Anthracnose) is a disease that infects strains of the genus *Colletotrichum,* causing black spots and necrosis on leaves, stems, and fruits. It is also a highly contagious disease that is difficult to eliminate and can cause economic damage. In this study, I confirmed that the *PepSTC* and *PepCYP* genes are related with the resistance against *Colletotrichum* in the leaves of pepper. In addition, I characterizated in molecular level that *PepSTC* and *PepCYP* are involved in the biosynthesis of a novel phytoalexin through transient expression in *N. benthamiana*. To understand the microenvironmental conditions for inducing *PepSTC* and *PepCYP* expression, various defense-related phytohormones were treated together with the inoculation of *C. coccodes*, a leaf-specific

pathogen. As a result of realtime PCR, the expression of both *PepSTC* and *PepCYP* increased when elicitor, methyl jasmonate and ethephon were treated simultaneously, which confirmed that the expression of both genes increased by synergistic effects of the defensive phytohormones and an elicitor.

To confirm the transient overexpression of *PepSTC* and *PepCYP* in *N. benthamiana*, a fluorescent protein was used to monitor the expression of the recombinant protein. Expression of the *PepSTC* and *PepCYP* genes was confirmed through gene and protein analyes, and subcellular localization of the two recombinant proteins was confirmed through fluorescence analysis. To confirm the function of *PepSTC* and *PepCYP* in phytoalexin biosynthesis, metabolites were extracted and analyzed from tobacco leaves expressing *PepSTC* and *PepCYP*. Substances were extracted from post infiltraed leaves and the effect on the resistance against *C. coccodes* in *N. benthamiana* was verified. As a result, a novel phytoalexin was biosynthesized in tobacco expressing *PepSTC* and *PepCYP*. Additionally, when both genes were expressed simultaneously, the accumulation of the phytoalexin increased. As a result of the expression of *PepSTC* and *PepCYP* in tobacco and inoculation with *C. coccodes*, the lesion diameter decreased compared to the control group. This result indicated that the resistance against *C. coccodes* in tobacco increase through the expression of *PepSTC* and *PepCYP*.

국문초록

형질전환된 담배에서 신규의 phytoalexin 합성에 관여하는 *PepSTC*와 *PepCYP* 유전자의 분자적 특성 규명

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식물탄저병(Anthracnose)은 *Colletotrichum* 속 균주의 감염으로 일어나며 잎, 줄기, 열매에서 검은 반점과 괴사현상을 일으키는 질병으로써, 전염성이 강하고 감염된 식물의 폐기가 어려워서 경제적으로도 큰 타격을 줄 수 있는 질병이다. 본 연구에서는 고추의 잎에서 *PepSTC*와 *PepCYP* 유전자를 통해 *Colletotrichum* 속 균주의 관계를 확인하고, 담배에 일시적 과발현을 통해 phytoalexin의 합성에서 두 유전자의 분자적특성을 규명하고자 하였다. 고추의 병 방어작용과 *PepSTC, PepCYP* 유전자의 관계를 확인하기 위하여 elicitor, 식물호르몬을 처리하였으며, 잎 특이적 병원균인 *Colletotirchum coccodes*를 접종하였다. Realtime PCR을 통해 *PepSTC*와 *PepCYP*의 발현을 확인한 결과 elicitor와 Methyl jasmonate를 통해 발현이 증가하였으며, Methyl jasmonate와 ethephon을 동시에 처리하였을 때 시너지 효과에 의해 두 유전자의 발현이 더욱 증가하는 것을 확인하였

다.

담배에서 *Agrobacterium*을 통한 *PepSTC*와 *PepCYP*의 일시적 과발현을 확인하기 위해 형광단백질을 이용하여 재조합 단백질을 발현시켰다. 두 유전자의 발현을 유전자, 단백질 분석을 통해 확인하였으며, 형광분석 방법을 통해 세포 내에서 두 유전자의 발현 위치를 확인하였다. Phytoalexin합성에서 *PepSTC*와 *PepCYP*의 기능을 확인하기 위해 물질을 추출하고, 담배에서 *C. coccodes*의 저항성에 미치는 영향을 확인하였다. *PepSTC*와 *PepCYP*를 발현시킨 담배의 잎에서 대사물질을 추출하여 분석한 결과 *PepSTC*와 *PepCYP*를 발현시킨 담배에서 대사물질이 유도되었으며, 두 유전자를 동시에 발현시켰을 때 대사물질의 축적량이 증가하였다. 또한 담배에서 *PepSTC*와 *PepCYP*를 발현시키고 *C.coccodes*를 접종하여 관찰한 결과 대조군에 비교하여 접종 부위의 면적이 감소하였다. 이 결과는 *PepSTC*와 *PepCYP*의 발현을 통해 담배 잎에서 *C. coccodes*의 저항성을 상승시킨 것으로 보여진다.

I. INTRODUCTION

Plants defend against pathogens through pre-existing defense barriers, like constitutively accumulated phytoanticipins, and induced the biosynthesis of phytoalexin (Garcion et al. 2014). Interestingly, these chemical defenses are classified into specific chemical characteristics according to plant groups (Takahashi et al. 2007). *Solanaceous* plants (potato, tobaco, tomato, pepper, etc.) synthesize sesquiterpenoid phytoalexin containing capsidiol, scopoletin (Zhang et al. 2005, Literakova et al. 2010).

Sesquiterpenes are natural products composed of a 15-carbon skeleton and have more than 7000 known compounds (Khorolragchaa et al. 2010, Fraga 2012). Many of these compounds have various functions as fragrance (Liu et al. 2021), antimicrobial (Yang et al. 2016), antibacterial (Jia et al. 2019) and antifungal (Taniguchi et al. 2014), and so on (Mai et al. 2021). Sesquiterpenes is catalyzed conversion of precursor farnesyl diphosphate (FPP) by a diverse range of sesquiterpene synthases including pentalenene synthase, germacrene C synthase, 5-epi-aristolochene synthase (EAS) and others (Cane et al. 1994, Back et al. 1998, Colby et al. 1998). *PepSTC* (EAS) has been reported as a key branch point enzyme for the synthesis of sesquiterpenoid phytoalexin in pepper leaves, is induced by UV-stress (Back et al. 1998). Furthermore, when *C. gloeosporioides* was inoculated into the ripe fruits, *PepSTC* gene expression and sesquiterpenoid phytoalexin accumulation were increased (Park et al. 2014). For the sesquiterpene production, two enzymes catalyzing consecutive steps in capsidiol biosynthesis are 5-epi-aristolochene synthase (EAS) and 5-epi-aristolochene dihydroxylase (EAH) belong to sesquiterpene cyclase and cytochrome P450 family,

respectively (Liang et al. 2021).

The cytochrome P450 (CYP) is a huge and important enzyme superfamily in plants, divided into 10 clans consisting of multiple family members (Nelson D R. 2006, Jun et al. 2015). The plant CYPs play diverse roles involved in plants growth and development (Khanom et al. 2019), regulation of phytohormones (Wang et al. 2020), and the biosynthesis process of secondary metabolites and phytoalexin (Geisler et al. 2013). The CYPs have been reported in *Arabidopsis*, tobacco, soybean, pepper, and other plants, and are still being studied for their characterization and functions. Among them, pepper cytochrome P450 (*PepCYP*) was highly expressed in the ripe stage in infected to pepper fruits that was inferred a relationship to the resistance against *C. gloeosporioides* (Oh et al. 1999). The amino acid sequence of PepCYP showed a hydrophobic membrane anchor in the N-terminal region and the heme domain of the PFGXGXRXCXG sequence in the C-terminal region. Additionally, *PepCYP* that belonged CYP71 family was highly homologous to Cytochrome 71D7 (*Capsicum chinense*, PHU24961.1), CYP71D8 (*Capsicum annuum*, KAF3625719.1), CYP71D20 (*Nicotiana tabacum*, NP_001311564.1). The sequence of the PepCYP protein showed 99.4 % homology with EAH (*Capsicum annuum,* KAF3619248.1) and 95 % identity to premnaspirodene oxygenase-like (*Capsicum annuum*, XP_016561857.1) (Figure 1).

Colletotrichum is known as the main cause of anthracnose disease and hosts various plants, including tropical, subtropical, and temperate fruits and crops (Dean et al. 2012). Among them, peppers are widely used economically and suffer an annual loss of more than 50% due to anthracnose, resulting in an economic loss of about 13% in Korea (Pakdeevaraporn et al. 2005). A typical symptom of anthracnose is necrotic sunken lesions

mixed with the acervuli. *Colletotrichum* specifications related to anthracnose have been reported as *C. acutatum*, *C. coccodes*, *C. dematium*, and *C. gloeosporiodes* (Than et al. 2008). While *C. coccodes* and *C. dematium* cause damage to leaves and stems, *C. acutatum* and *C. gloeosporioides* are infected with fruits (Oo and Oh 2016).

Although anthracnose diseases have been well-studied in pepper fruits, additional research is needed in vegetative organs to understand the underlying mechanisms responsible for the disease resistance (Saxena et al. 2016). Much of what is known concerning the interaction of *Colletotrichum* species with pepper hosts is drawn from two species; *C. gloeosporioides* and *C. capsici*, which cause anthracnose symptoms on the fruits (Ko et al. 2005, Prasath and Ponnuswami 2008). However, it was reported that *C. coccodes*, the causative agent of leaf anthracnose, led to extensive loss of pepper plants in 1998, and also resulted in damage to many crops, such as potatoes and tomatoes (Hong and Hwang 1998, Lees and Hilton 2003). Accordingly, I tried to examine the biosynthesis of sesquiterpenoid phytoalexin during the interaction between the pepper leaves and *C. coccodes*. Furthermore, since *PepCYP* showed the highest homology with EAH, the penultimate enzyme, pepper sesquiterpene cyclase (*PepSTC*) gene, was used together to confirm the reciprocal relation between the two genes, and to analyze the production of the enzymatic reaction product.

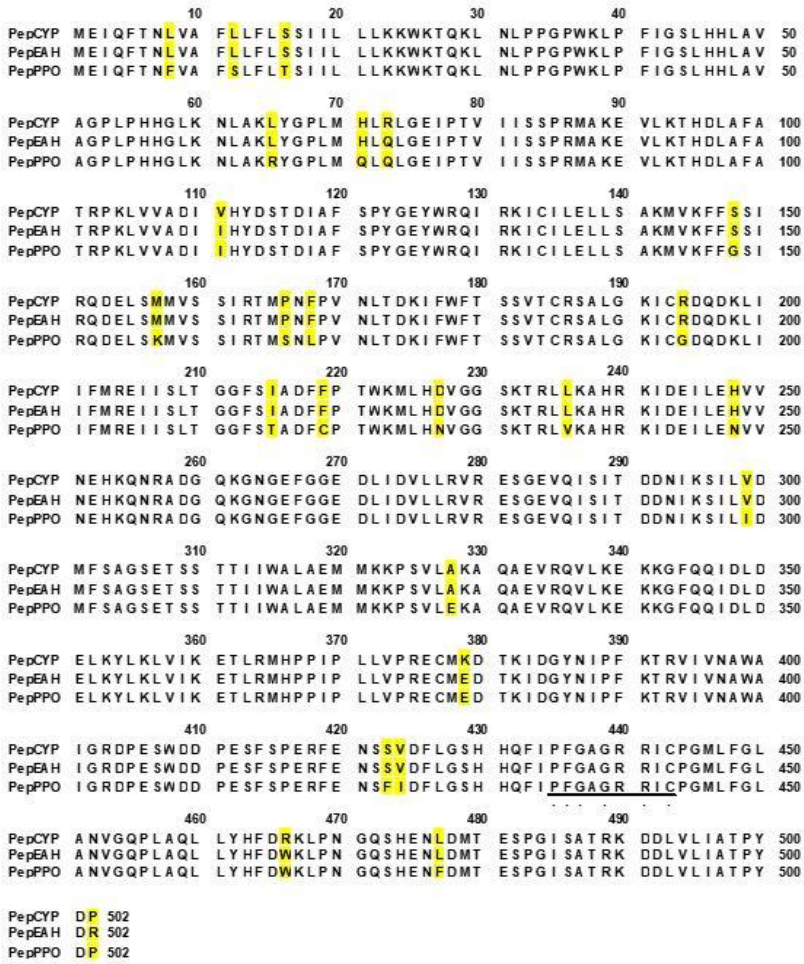

Figure 1. Amino acid alignment of PepCYP (AAF27282.1) with other pepper cytochrome P450. 5-epi-aristolochene 1,3-dihydroxylase, 99.4% (PepEAH, *Capsicum annuum*, KAF3619248.1), premnaspirodiene oxygenase-like, 95% (PepPPO, *Capsicum annuum*, XP_016561857.1). The PFGXGXRXCXG heme-binding domain, a sequence indicated by dots, was conserved in the C-terminal region.

II. MATERIALS AND METHODS

1. Plant and fungal materials and inoculation

Korean chill pepper (*Capsicum annuum* L.) and *Nicotiana benthamiana* were grown at 25±2℃ in a growth room with a photoperiod of 16 light/8 dark. For all pepper samples, 6-8 leaf stage peppers were used, and in the case of pepper fruits, they were detached from 2-3 month-old plants. *N. benthamiana* is grown for 4-5 weeks, and before infiltration, the plants were placed in a dark and high humidity state. *Colletotrichum coccodes* (KACC 40009) were obtained from Korean Agricultural Culture Collection (KACC). *C. coccodes* was maintained on PDA (potato dextrose agar). The conidial suspension was obtained from the 7-10 days-old cultures on the plate. Conidia were harvested by spraying sterile water with a pipet. The inoculum concentration was adjusted to 5×10^5 conidia mL^{-1} with sterile water, and add 0.05% tween 20. Pepper leaves were inoculated by pipetting 10 μL droplets on the adaxial surface between leaf veins(Hong et al. 2001). Inoculated pepper leaves were then placed in an acrylic box that was moistened and sealed tightly to maintain relative humidity near 100% and in the dark state, after which they were incubated at 25℃.

2. Eilcitor and phytohormone treatment

The expression of pepper genes (*PepSTC*, *PepCYP*) induced by phytohormones was analyzed by realtime PCR with table 1 primer. Leaves were harvested from 8-stage-pepper and treated 0.5% cellulase, 100 μM methyl-jasmonate, and 100 μM ethephon by pipetting 10 μL droplets on the adaxial surface between leaf veins (Jin, Zhang et al. 2019). The

leaves were then placed in an acrylic box that was moistened and sealed tightly to maintain relative humidity near 100%, after which they were incubated dark state at 25℃.

3. Reverse transcription PCR and realtime PCR

Total RNA was isolated from the sample powder with Apure® Plant RNA kit (GenomicsBase co ltd, Seoul, Korea) and used as the template for reverse transcription, using M-MLV Reverse Transcriptase (Promega, Madison, USA). For expression analysis of *PepSTC* and *PepCYP* genes, RT-PCR was performed with AccuPower® PCR Premix (bioneer Corp., Korea) and quantified through quantitative realtime PCR using RealAmp SYBR™ qPCR Master mix (GeneAll, Korea) with table 1 primer. The data were analyzed using the Rotor gene 6 software, and the *UBQ* gene was used to reference the gene for normalization.

4. Immunoblot analysis

Total proteins were extracted from 100 mg sample powder with 200 μL extract buffer (50 mM sodium phosphate buffer, 10 mM EDTA(pH 8.0), 0.1% Triton X-100, 0.1% SDS, 25 mM Sucrose, 10% glycerol) containing PMSF, inhibitor cocktail for 3 hours (h) on ice and then centrifuged at 12,000 rpm for 10 min. Protein concentrations were measured by the BSA assay kit. Proteins were then analyzed by immunoblot analysis with specific antibodies for PepSTC, PepCYP, Actin, and Ubiquitin for 16 h at 4℃. The appropriated horseradish peroxidase-conjugated Anti-mouse and anti-rat were used as secondary antibodies. Band detection was used for the SuperSignal™ West Femto Maximum Sensitivity Substrate detection system and exposed to X-ray films.

5. Plasmid construction

The expression vector pGC5 was constructed with T4 ligase and restriction enzyme. The plasmid was inserted into an *Xba* Ⅰ/*Hind*Ⅲ, *EcoR* Ⅰ/*Sac* Ⅰ-digested fragment of CaMV 35S promoter and Nos terminator sequence from pBI121 into pCAMBIA1300. 1.5 Kb cDNA of *PepCYP* (GenBank accession no. AF122821.1) and 1.68 Kb cDNA of *PepSTC* (GenBank accession no. AF061285) were amplified by PCR.

6. *Agrobacterium* mediated transient expression in *N. benthamiana*

Plant expression vectors were transformed into the *Agrobacterium tumefaciens* GV2260 strain by the freeze-thaw method (Deblaere et al. 1985). The *Agrobacterium* was streaked on a YEP agar plates containing rifampicin (50 μg mL^{-1}) and kanamycin (50 μg mL^{-1}). The plates were incubated at 28℃ for 2-3 days, and the colony was selected by PCR. The selected colony was cultured at 180 rpm in 3 mL of YEP medium containing antibiotics at 28℃ for 2 days. The pre-cultured *Agrobacterium* was subcultured at 28℃ in 10 mL YEP medium containing 10 mM MES (pH 5.6), 100 μM acetosyringone, and the antibiotics until OD_{600} = 0.8. After the suspension was centrifuged at 4,000 rpm and removed medium, the pellet was adjusted at OD_{600} = 0.2 in Infiltration buffer (10 mM $MgCl_2$, 10 mM MES (pH 5.6), 100 μM acetosyringone) and incubated at 28℃ for 1 h 30 min. Co-infiltration, the suspension was adjusted OD_{600} = 0.4 and mixed at 1:1 (v/v) ratio. The suspension was infiltrated into the abaxial side of *N. benthamiana* leaves with a 1 mL non-needle syringe and incubated under high humidity and dark conditions for 24 h. It was then transferred to the growth room and used in each experiment (Brückner and Tissier

2013). Expressions of *PepSTC* and *PepCYP* were confirmed by realtime PCR and western blot. Leaves of post-3 days infiltrated *N. benthamiana* were monitored by confocal laser-scanning microscopy (LSM 510, Carl Zeiss).

7. Thin layer chromatography of the metabolic extracts

The sample powder (300 mg) was homogenized in 1 mL of 50% methanol. The mixture was centrifuged at 16,000 rpm for 5 min, and the supernatant was mixed with 1 mL of ethyl acetate/cyclohexane (1:1, v/v). The mixture was centrifuged at 3,000 rpm for 5 min, and the upper phase (ethyl acetate layer) was collected and dried up with nitrogen gas. The resultant precipitate was suspended in 15 μL of methanol and subjected on TLC plate. Extracted samples (15 μL lane) were developed on TLC plates (25 TLC aluminum sheets silica gel 60; Merck) with ethyl acetate/cyclohexane (1:1, v/v) and visualized with a staining solution (2.8% vanillin, 0.5% sulfuric acid in methanol), followed by heating at 120℃ (Matsukawa et al. 2013).

8. Qualitative analysis of metabolic extracts by GC-MS

The identification of *N. benthamiana* leaves extracts were performed by Gas chromatograph (GC-MS, SHIMADZU, Japan) and separated with Rtx-1 capillary column (30 m × 0.2 mm ID, 0.25 μm df). The column was programmed as follows: initial temperature of 100℃, followed by an increase to 300℃ over 20 min (10℃ min^{-1}), where the temperature was maintained for 5 min. The samples were injected 1 μL with AOC500 (auto injector) at 180℃. Helium was applied as the carrier gas at a flow rate of 1 mL min^{-1}. The interface space and ion source were maintained at 280℃ and 300℃, Mass

range was programmed 50-500 m/z (Park et al. 2014).

9. Antifungal activity of phytoalexin against *C. coccodes*

The sample powder (15 g) was homogenized in 375 mL of 50% methanol (1:25). The mixture was centrifuged at 3,000 rpm for 5 min, and the supernatant was mixed with 375 mL of chloroform (1:1, v/v). The mixture was separated by fractionation, and the down phase (chloroform layer) was collected and dried up with a rotary evaporator and nitrogen gas. The resultant precipitate was suspended in 500 μL of 100% ethanol. Extracted samples were separated on TLC plate (25 TLC silica gel 60; Merck) with ethyl acetate/cyclohexane (1:1, v/v) and harvested the phytoalexin from the TLC plate at Rf = 0.475 region.

Following a phytoalexin extraction, the antifungal activity of the phytoalexin was examined against *C. coccodes*. 5 μL of phytoalexin in 5% DMSO (to a final concentration of 0.125, 0.25, 0.5 and 1 mM) was added to 5 μL of conidia in sterile distilled water (4×10^5 conidia mL^{-1}) on a cover glass. As mock and control, 5 μL of sterile distilled water or 5% DMSO was added to the same amount of conidia suspension. The cover glasses were incubated in a humidified chamber at 25℃ for 24 h. At 24 h after phytoalexin treatment, conidia germination and appressorium formation of *C. coccodes* were observed and, counted using a microscope.

10. Enhanced resistance of *N. benthamiana* transiently over-expressing *PepSTC* and *PepCYP* against *C. coccodes*

To investigate the efficacy of phytoalexin against *C. coccodes* in *N. benthamiana* leaves. *PepSTC* and *PepCYP* were transiently expressed in *N. benthamiana* leaves by *Agrobacterium* infiltration, and the *N. benthamiana* leaves were inoculated with *C. coccodes*. Three transgenic *N. benthamiana* lines (*PepSTC*, *PepCYP*, and *PepSTC+PepCYP*) were evaluated for resistance to *C. coccodes*, and wild type *N. benthamiana* was used as the positive control. At 3 days after infiltration, the *N. benthamiana* leaves were detached from each transgenic line. 10 μL of conidia suspension (5×10^5 conida mL^{-1}) in distilled water was inoculated on the surface of *N. benthamiana* leaves, as described previously (Hong et al. 20201) with the following modification. The infected leaves were placed in an acrylic box and incubated at 25℃ for 6 days. The disease symptoms were monitored until 6 days after infection. The infected leaves were decolorized with 100% ethyl alcohol and stained with 1% lactophenol blue to observe the fungal hyphae under a microscope. The response of *N. benthamiana* leaves to infection was determined by measuring the lesion diameter.

11. Statistical analysis

Experimental data were subjected to analysis of variance (ANOVA) using IBM SPSS statistics software. Significant differences between mean values were determined at $p < 0.05$. All data were presented as the means ± SD of at least three independent experiments.

Table 1. A list of primer sequences used in this study.

Name	Sequence	Use
STC-Kpn1-IF-Rev	TGC TCA CCA TCC CGG GTA CCT GAA TTT TGA ACG AGT CAA CAA CCA TG	Vector Construct
Kpn1-STC-IF-For	CGG GGG ACG AGC TCG GTA CCA TGG CCT CAG TTG CAG TTG	
PepCYP-BamH1-Rev	CGG GAT CCA GGA TCA TAA GGG GTG GCA AT	
PepCYP-Xba1-For	GCT CTA GAA TGG AGA TTC AAT TCA CCA ACT TAG TTG	
STC-RT-Rev	GAA CGA GTC AAC AAC CAT GGC	RT-PCR
STC-RT-For	CGC AAA AGA AAG ACT GAA AGA ATT GG	
PepCYP-RT-Rev	GGT GGC AAT CAA AAC AAG ATC ATC C	
PepCYP-RT-For	GCT GGA TCT GAA ACG TCA TCG	
pepper_UBQ-RT-For	CAT GGG GCC GAC AGA TAG	Realtime PCR
pepper_UBQ-Rev	CAC CCC AAG CAC AAT AAG AC	
PepCYP-qRTPCR-For	GCA TGG GCA ATT GGA CGA G	
PepCYP-UTR3-Rev	CAA CAA AAA TGA CCT GGA AGC AG	
PepSTC-qRTPCR-For	GGT GAG TCG GGA TGG AAG G	
PepSTC-UTR-3-Rev	GCA AAG ACA ACA CAA ACA TCT ATG AG	

III. RESULTS

1. *PepSTC* and *PepCYP* expression in various healthy tissues of pepper

PepSTC and *PepCYP* are expected to be involved in phytoalexin biosynthesis during plant-pathogen interactions. I confirmed *PepSTC* and *PepCYP* in healthy tissues before examining the expression of *PepSTC* and *PepCYP* in the defense relationships of pepper leaves. Total RNA was extracted from the flower, flower bud, stem, leaves, root, green fruit, and red fruit and performed RT-PCR and realtime PCR by using the specific primers. Figure 1 shows that *PepSTC* and *PepCYP* were hardly expressed in the healthy tissues, except for roots showing weak expression.

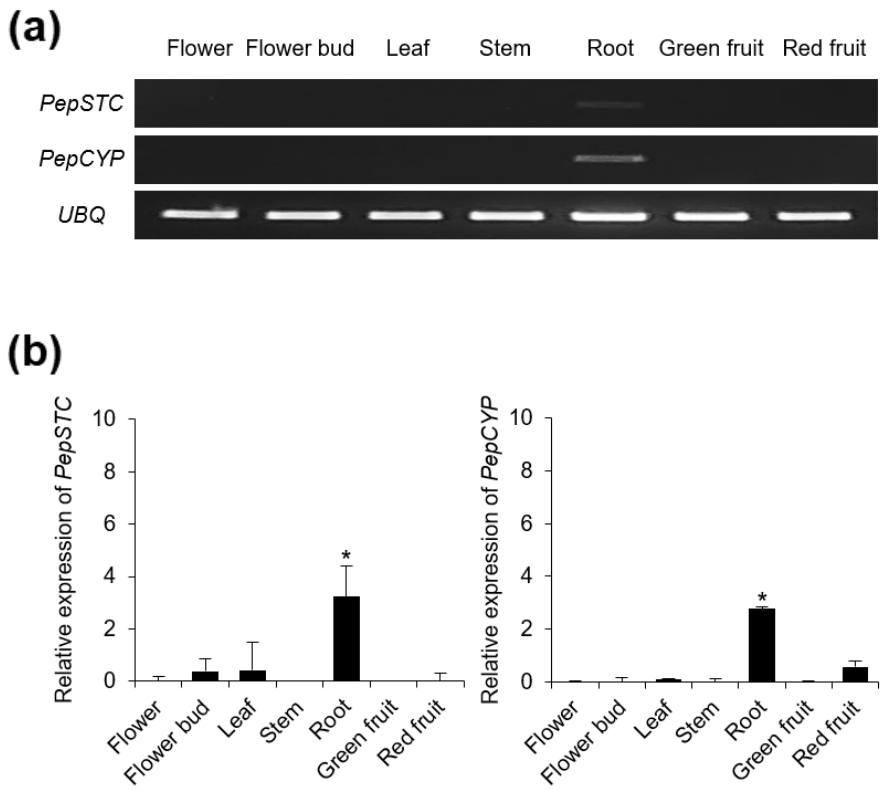

Figure 2. *PepSTC* and *PepCYP* expression in various healthy tissues of pepper. Each organ was sampled from 8-leaf-stage pepper, and fruits were sampled 2-3 months after germination. **(a)** Reverse transcription PCR. **(b)** Relative expression levels of *PepSTC* and *PepCYP*, normalized by different candidate reference genes (*UBQ*). ANOVA was conducted to assess significant differences and the asterisks above the bars indicated statistically significant differences regarding the lowest expression organ (flower) ($p < 0.05$).

2. Induction of *PepSTC* and *PepCYP* genes by elicitor and phytohormones treatment.

To examine the relationship between disease resistance and plant hormones, pepper leaves were treated with cellulase, methyl jasmonate, and ethephon. Total RNA was extracted from cellulase, methyl jasmonate, ethephon treated pepper leaves for 3, 24, and 48 h and I performed realtime PCR. As a result of cellulase treatment, *PepSTC* and *PepCYP* were induced 3 h after treatment. Then, the expression of *PepSTC* decreased after 24 h, but the expression of *PepCYP* was maintained until 48 h. Upon treatment methyl jasmonate, expression of *PepSTC* was increased 24 h and maintained until 48 h, and the expression of *PepCYP* increased 3 h and gradually decreased until 48 h. Next, after the treatment with Ethephon, both *PepSTC* and *PepCYP* showed induced expression only 3 h after treatment. However, when the leaves were treated with both methyl jasmonate and ethephon at the same time, their expression patterns was similar by revealing at higher induction at 3 h with gradual decrease, but the expression levels were magnificently higher, especially in STC. Our results suggest that the induction of the sesquiterpene pathway could be regulated by the jasmonate and ethylene signaling pathway.

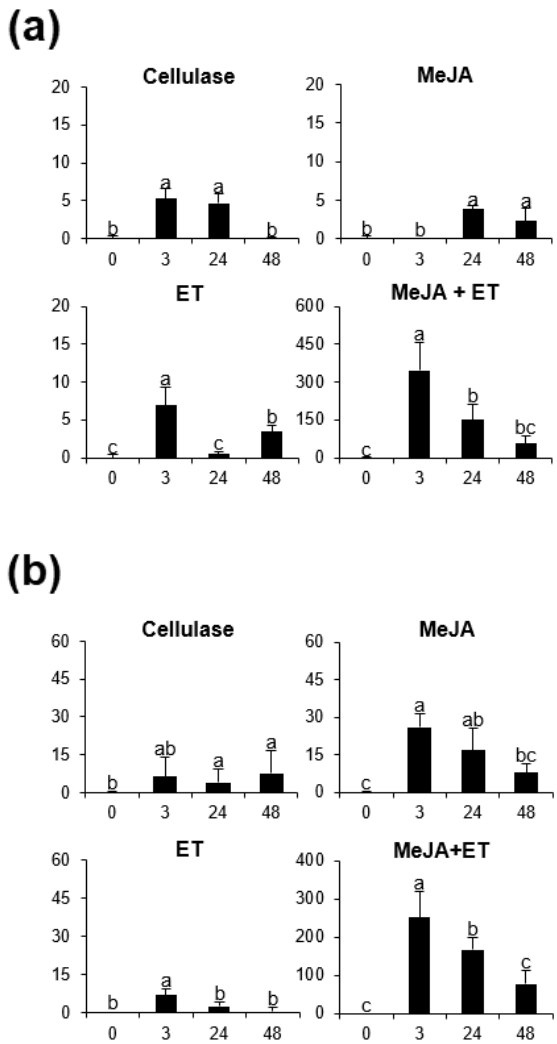

Figure 3. Induction of *PepSTC* and *PepCYP* genes by elicitor and phytohormone treatment. Total RNA was extracted from samples 3, 24, and 48 h after hormone treatment on pepper leaves, and 0.5% cellulase treatment was used as a positive control. Relative expression levels of *PepSTC* (a) and *PepCYP* (b), was normalized by different candidate reference genes (*UBQ*). Means with different letters (a-c) are statistically significant as determined by Turkey's multiple tests ($p < 0.05$). MeJA; Methyl jasmonate, ET; Ethephon.

3. Expression of *PepSTC* and *PepCYP* genes by fungal inoculation

The relationship between *PepSTC* and *PepCYP* gene was analyzed by inoculating *C. coccodes* on pepper leaves. Then, total RNA and protein were extracted from inoculated leaves obtained at each period. The expression of each gene was shown in Figure 4 by realtime PCR and Western blot. As shown in Fiugre 4b, the expression of *PepSTC* was increased 12 h after fungal inoculation and maintained until 24 h, but decreased after 48 h. *PepCYP* increased expression until 24 h and decreased after 48 h. Also, result of the western blot (Figure 4c), the PepSTC and PepCYP proteins were confirmed 3 h after fungal inoculation and maintained until 24 h, but decreased after 48 h. The result showed that the expression of *PepSTC* and *PepCYP* were involved initial defense response against *C. coccodes*

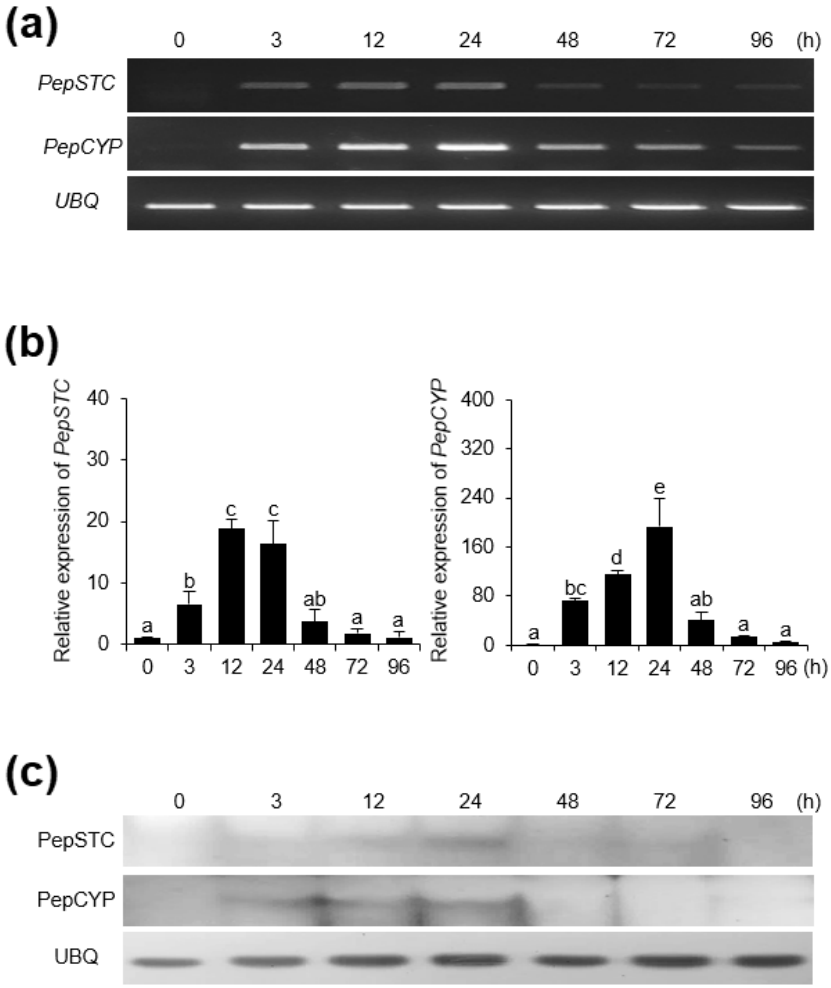

Figure 4. The expression of *PepSTC* and *PepCYP* genes in pepper leaves by *C. coccodes* inoculation was analyzed. Total RNA and protein were extracted from samples 3, 12, 24, 48, 72, and 96 h after being inoculated with *C. coccodes* on pepper leaves. (a) Reverse transcription PCR. (b) Relative expression levels of *PepSTC* and *PepCYP* normalized by different candidate reference genes (*UBQ*). Means with different letters (a-e) are statistical significance as determined by Turkey's multiple tests ($p < 0.05$). (c) Western blot.

4. Expression of *PepSTC* and *PepCYP* in the leaves of agro-infiltrated *N. benthamiana* and subcellular localization

For the transient over-expression of *PepSTC* and *PepCYP* in *N. benthamiana*, constructs were prepared in the binary vector containing the 35s promoter and Nos terminator (Figure 5). In addition, subcellular localization was observed by expressing a recombinant protein fused with fluorescent proteins when the constructs were introduced into *N. benthamiana* leaves by agro-infiltration. Then, *N. benthamiana* leaves were collected at each period to confirm the expression of the genes (Figure 6). As a result, the *PepSTC-eYFP* was expressed and maintained in the leaves until the fifth day after infiltration, and *PepCYP-eGFP* expression was observed on the third day and decreased gradually thereafter. When both genes were expressed at the same time, it was confirmed that the highest expression was shown on the third day and decreased thereafter.

Therefore, subcellular localization was observed through confocal microscopy on the 3rd day (Figure 7). As a result, it was observed that PepSTC-eYFP was expressed in the cytoplasm. Meanwhile, PepCYP-eGFP showed a specific intracellular expression pattern in the membrane structure. To verify this, the site of expression of PepCYP protein was confirmed to be ER (Endoplasmic reticulum) through the Plant-mPLoc program. Expression patterns were compared using BiP (Binding immunoglobulin protein) as an ER protein marker (Figure 8) (Zalán Czékus et al. 2022). As a result of comparing the expression patterns, it was confirmed that the expression pattern was the same as that of BiP protein, an ER marker. Through this, it was confirmed that *PepSTC* and *PepCYP* were transiently overexpressed in *N. benthamiana*, and PepSTC and PepCYP protein were observed to be expressed in the cytoplasm and endoplasmic reticulum, respectively.

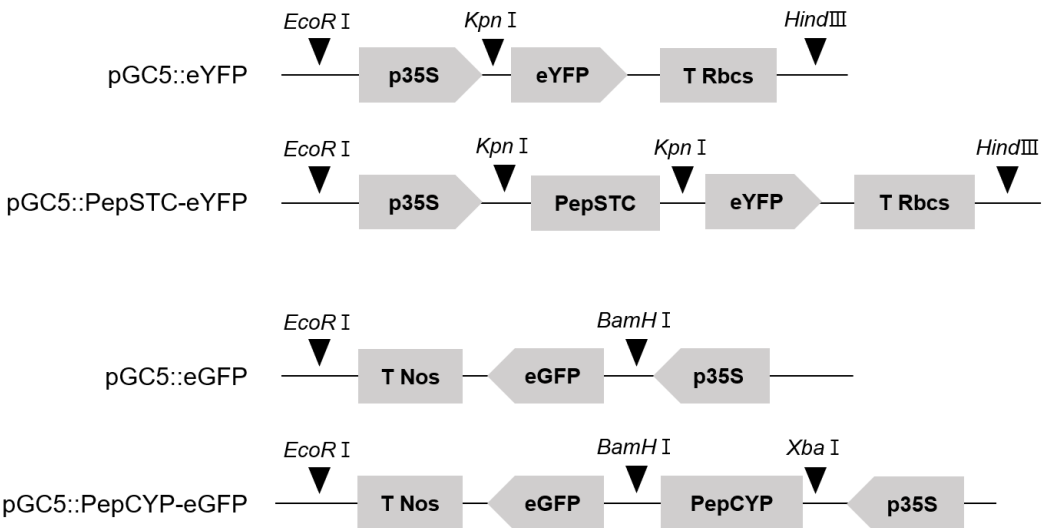

Figure 5. Schematic representation of plant expression vector for *Agrobacterium* mediated infiltration. p35S; Cauliflower mosaic virus (CaMV) 35S promoter, T Nos; nopaline synthase terminator, eYFP; enhanced yellow fluorescent protein, eGFP; enhanced green fluorescent protein, T Rbcs; rubisco synthase terminator.

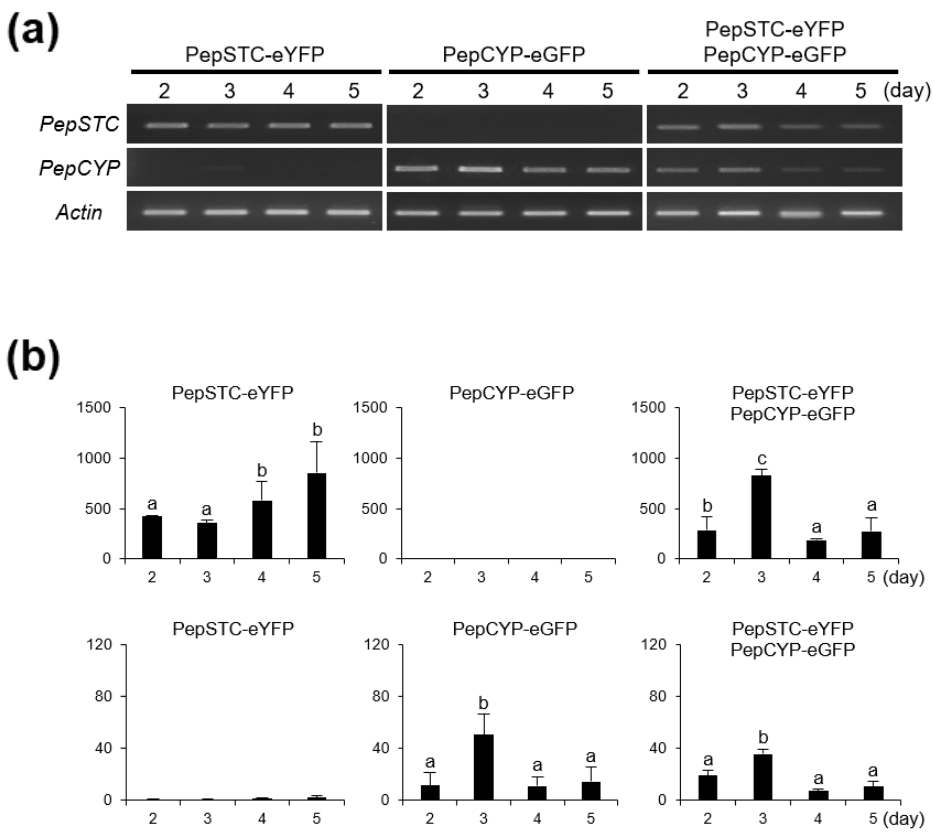

Figure 6. Analysis of pepper genes (*PepSTC* and *PepCYP*) transiently expressed in *N. benthamiana*. Total RNA was extracted from infiltrated leaves of *N. benthamiana*. (a) Reverse transcription PCR. (b) Relative expression levels of *PepSTC* (upper) and *PepCYP* (under), normalized by different candidate reference genes (*Actin*). Means with different letters (a-c) are statistical significance as determined by Turkey's multiple tests ($p < 0.05$).

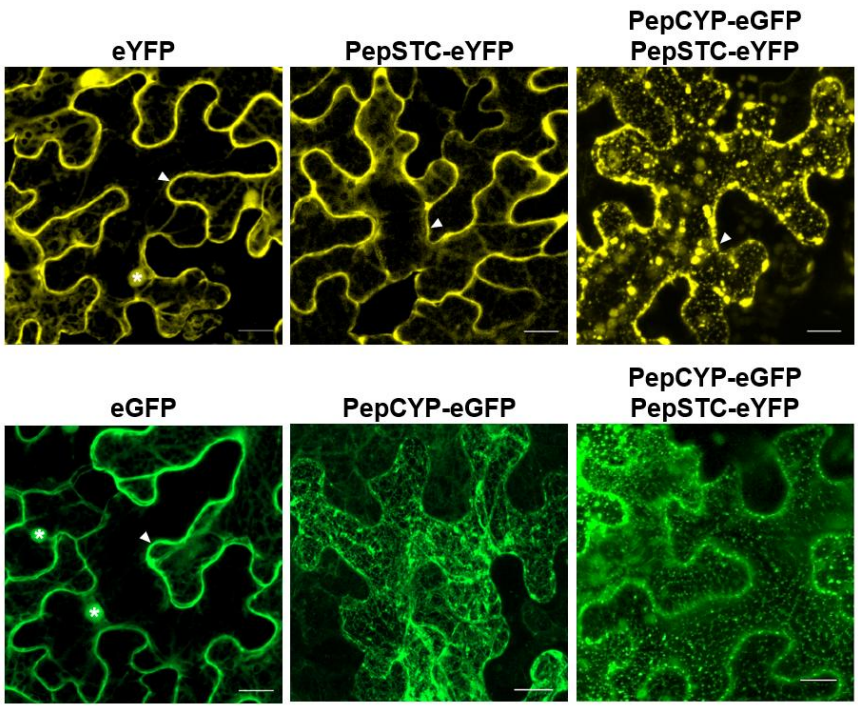

Figure 7. Subcellular localization of PepSTC-eYFP and PepCYP-eGFP in epidermal cells of infiltrated *N. benthamiana*. Confocal microscopy scans were performed 3 days post-infiltration in *N. benthamiana*. Asterisks indicate the nucleus and arrows indicate cytoplasm. Bars = 10 μm.

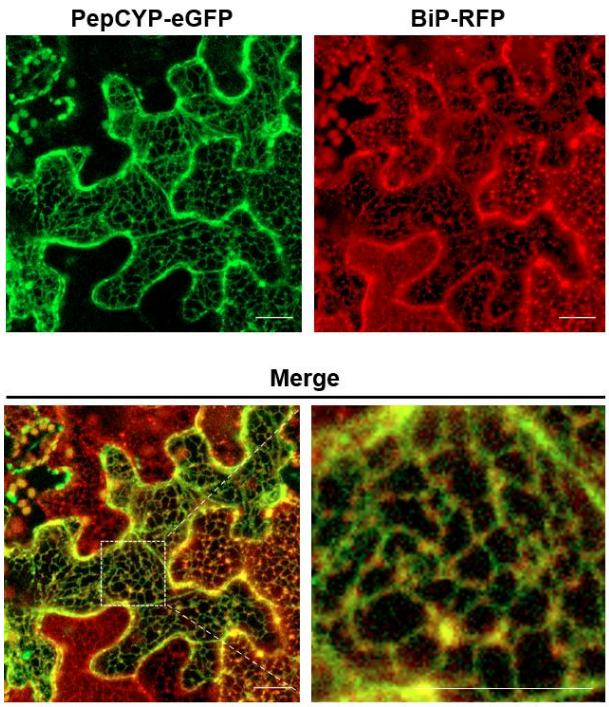

Figure 8. Structure formation and intracellular distribution of transiently expressed of the fusion protein (PepCYP-eGFP and BiP-RFP). Confocal microscopy scans were performed 3 days after infiltration in *N. benthamiana*. The BiP-RFP was used as endoplasmic reticulum (ER) marker by co-infiltration. BiP; Binding immunoglobulin protein. Bars = 10 μm.

5. Analysis of tobacco leaf extracts transiently over-expressing *PepSTC* and *PepCYP*

In a previous experiment, I confirmed that the transient expression of *PepSTC* and *PepCYP* gene was induced in *N. benthamiana* by Agro-meditated infiltration. Therefore, to identify the metabolites induced by the expression of *PepSTC* and *PepCYP*, metabolites were extracted from the transiently expressed leaves of *N. benthamiana*. As shown in Figure 9A, the expression of *PepSTC* and *PepCYP* was confirmed by realtime PCR and western blot. (Figure 9b, c).

The metabolites were extracted by adding 50% methanol to the sample powder, followed by fractionation with ethyl acetate/cyclohexane. Then, I harvested the ethyl acetate layer (upper), concentrated it with nitrogen gas, and then developed it on a TLC plate. As a result, it was confirmed that unlike the *eGFP* sample used as a control, the substance at Rf = 0.475 was accumulated in the samples expressing *PepSTC* and *PepCYP*, and more accumulation was induced when both genes were expressed at the same time (Figure 10). Next, as a result of GC-MS, the *eGFP* sample did not induce substance accumulation as shown in the TLC plate analysis result, but the substance at Rt = 12.93 was accumulated in the *PepSTC* and *PepCYP* samples, and more accumulation was induced when both genes were expressed at the same time (Figure 11). As a result of this experiment, it confirmed that the expression of *PepSTC* and *PepCYP* induced the accumulation of a substance when expressed in both genes at the same time.

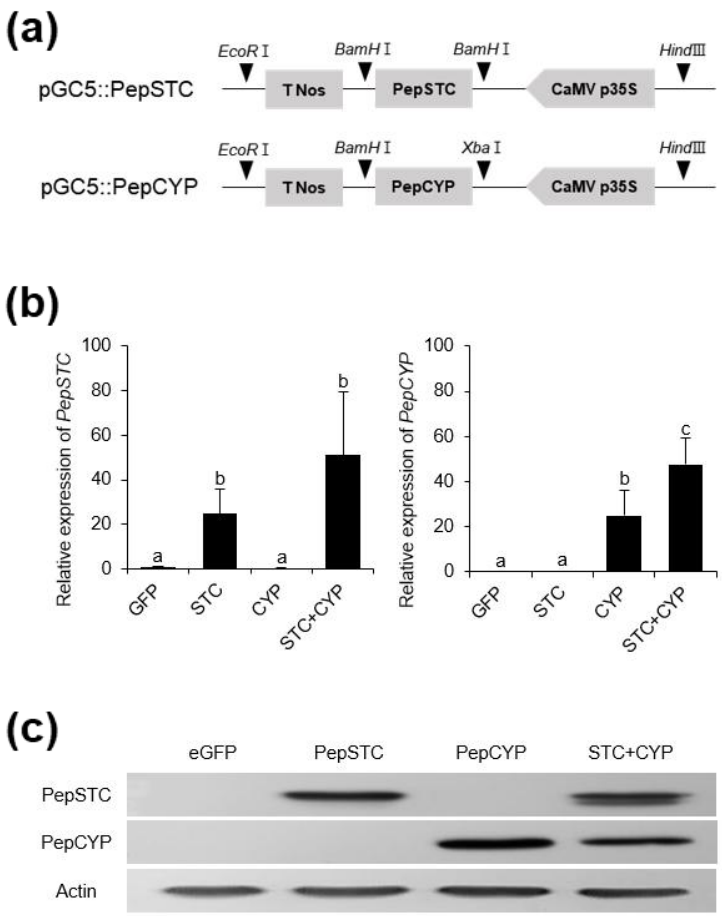

Figure 9. Transient expression of pepper genes (*PepSTC* and *PepCYP*) in infiltrated *N. benthamiana*. (a) Vector constructs for transient expression. (b) Relative expression levels of *PepSTC* and *PepCYP* normalized by different candidate reference genes (*Actin*). (c) Western blotting of post-infiltration leaves of *N. benthamiana*. Total RNA and protein were extracted 3 days after the infiltration sample. PepSTC and PepCYP proteins were detected the specific antibodies. Means with different letters (a-c) are statistical significance as determined by Turkey's multiple tests ($p < 0.05$).

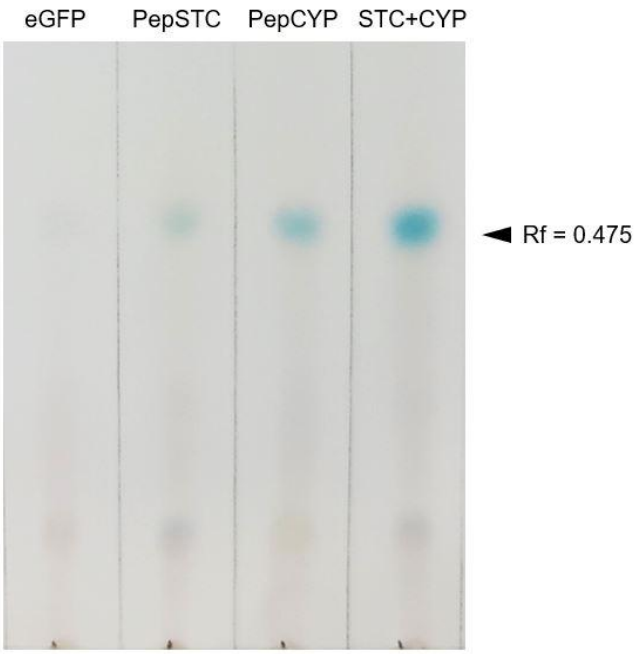

Figure 10. Analysis of total extracted metabolite by TLC. Metabolites were extracted 5 days after infiltration. The samples were separated and developed on a thin layer chromatography plate. Target spots are indicated by an arrowhead. Rf; Retention factor.

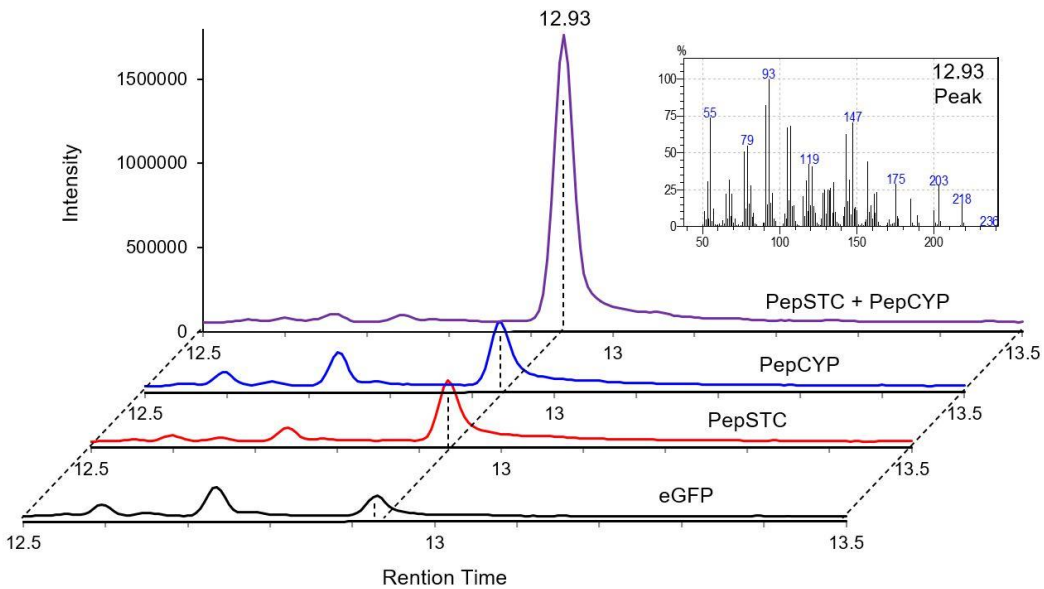

Figure 11. Gas chromatography-Mess spectrometry (GC-MS) profile of methanolic extracts of 5 days post-infiltrated *N. benthamiana*. Co-infiltration (*PepSTC+PepCYP*) is to confirm the differences by the interaction of pepper genes.

6. Antifungal activity of phytoalexin against *C. coccodes*.

To confirm the antifungal activity of phytoalexin against anthracnose fungus, *C. coccodes*. I treated with 0.125, 0.25, 0.5, and 1 mM phytoalexin dissolved in 5% DMSO to conidia suspension of *C. coccodes* and observed to evaluate conidia germination and appressorium formation using a microscope.

In the control sample that was treated with 5% DMSO, the fungal growth observed the usual conidia germination and appressorium compared with the mock sample (distilled water treatment). The treatment with 0.125, 0.25 mM phytoalexin did not affect conidia germination and appressorium formation in *C. coccodes* (Figure 12a). However, the phytoalexin-treated fungus were appeared without appressorium formation at 1 mM. Additionally, the phytoalexin application at 1 mM had a 50% inhibitory effect on conidia germination of *C. coccodes* and the appressorium formation rate was significantly reduced compared with the control (Figure 12b). These results indicate that the phytoalexin has antifungal activity against *C. coccodes*.

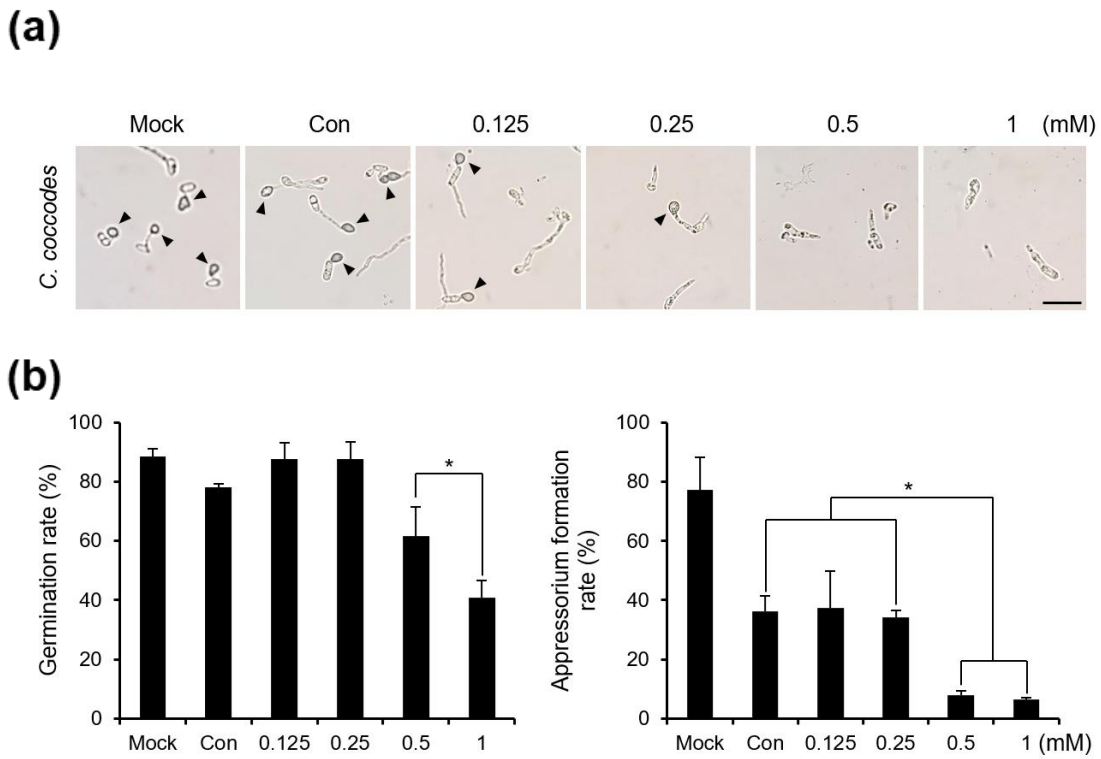

Figure 12. Inhibitory effects of phytoalexin on the growth of *C. coccodes*. Evaluation of conidia germination and appressorium formation by phytoalexin treatment. The conidia were amended on cover glass with 10 µL of phytoalexin dissolved in 5% DMSO at concentrations of 0, 0.125, 0.25, 0.5, 1 mM, then incubated for 24 h. Bar = 20 µm. (a) Observed phenotypes. (b) Conidia germination and appressorium formation. ANOVA was conducted to assess significant differences and the asterisks above the bars indicated statistically significant differences ($p < 0.05$). Mock; distilled water treatment Con; 5% DMSO treatment. The arrows indicated as appressorium of *C. coccodes*.

7. Transient expression of Pepper genes (*PepSTC* and *PepCYP*) improved resistance against *C. coccodes* in *N. benthamiana.*

To further examine the level of disease resistance in *N. benthamiana* leaves were expressed with *PepSTC* and *PepCYP*. As a result, control *N. benthamiana* leaves showed the disease symptoms in which extensive fungal hypha and distinct lesions were observed 6 days after inoculation (Figure 13a). Additionally, *PepSTC* or *PepCYP* transgenic plants also well observed elongation of hypha on infected leaves. However, the hypha growth of *C. coccodes* was inhibited in *N. benthamiana* leaves over-expressing the *PepSTC* and *PepCYP*. Also, compared with the control plants, the lesion diameter was significantly reduced to approximately 50% in the *N. benthamiana* plant over-expressing the *PepSTC* and *PepCYP* (Figure 13b). These results indicate that over-expression of *PepSTC* and *PepCYP* in *N. benthamiana* leads to increased phytoalexin accumulation and effectively suppressed the hyphae growth of *C. coccodes*. Thus, co-expression of the *PepSTC* and *PepCYP* improves resistance in the transgenic *N. benthamiana* plants against *C. coccodes.*

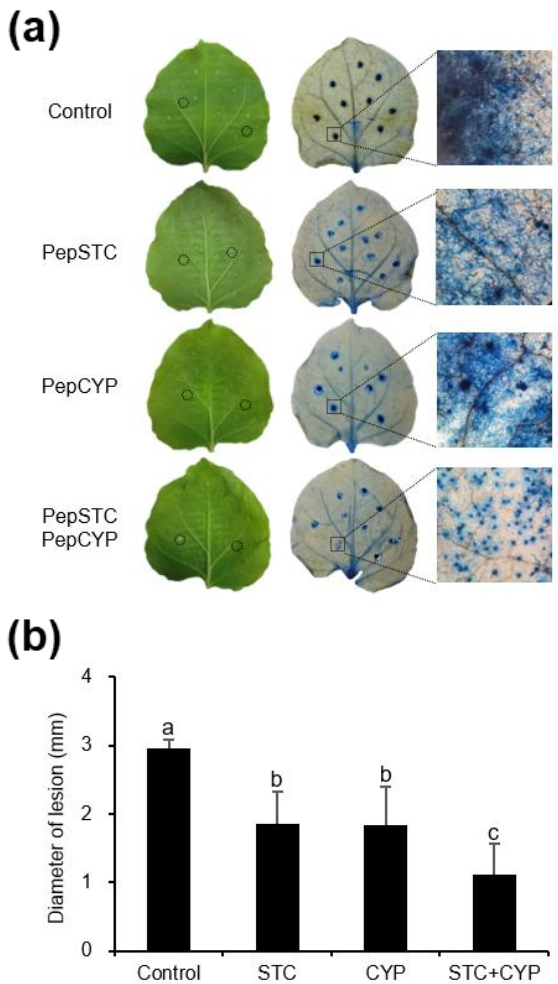

Figure 13. Transient expression of Pepper genes (*PepSTC* and *PepCYP*) improved resistance against *C. coccodes* in *N. benthamiana*. 10 μL of *C. coccodes* 5×10^5 conidia mL^{-1} were inoculated in the infiltration regions 3 days post infiltrated with the indicated genes and the photograph was then taken 6 days post-inoculation and stained with lactophenol blue. (a) Observed phenotypes. (b) Lesion diameters were measured using the ImageJ program. Means with different letters (a-c) are statistical significance as determined by Turkey's multiple tests ($p < 0.05$). Circles indicated as infiltration spots.

IV. DISCUSSION

In this study, I investigated the relationship between *PepSTC* and *PepCYP* in biosynthesis of sesquiterpenoid phytoalexin between the pepper leaves and *C. coccodes*. *PepSTC* is induced in the red pepper fruits during fungal infection and is known as a sesquiterpene cyclase for sesquiterpenoid phytoalexin biosynthesis (Park et al. 2014). PepCYP protein contains a hydrophobic membrane anchor and heme-binding domain, the *PepCYP* gene product plays a critical role in the plant defense mechanism against the fungal invasion and colonization of fruit in the incompatible interaction (Oh et al. 1999). Sequence comparison showed that the PepCYP protein shared the highest homology to 5-epi-aristolochene 1,3-dihydroxylase (EAH) (Figure 1). 5-epi-aristolochene synthase (EAS) and 5-epi-aristolochene hydroxylase (EAH) were key enzymes for sesquiterpenoid phytoalexin biosynthesis (Song et al. 2019).

Our data showed that *PepSTC* and *PepCYP* did not express in healthy tissue of pepper (Figure 2). Also, the combination treatment of methyl jasmonate and ethephon increased the expression of *PepSTC* and *PepCYP* in pepper leaves (Figure 3). These results showed that healthy pepper do not express the phytoalexin pathway in leaves and the expression of *PepSTC* and *PepCYP* was related to jasmonate and ethylene signaling pathway. It was reported that when *N. benthamiana* was inoculated with *Phytophthora infestans*, pathogen-induced *NbEAS* and *NbEAH* expression were abolished in plants with ethylene insensitive 2 (*NbEIN2*) silenced, suggesting that the genes are related to the ethylene signaling pathway (Song e al. 2019). Jasmonic acid and ethylene up-regulate the expression of scores of defense-related genes including cytochrome P450 (Reymond et al. 1998). Both

the jasmonate and ethylene signaling pathways are required for the activation of plant defense against necrotrophic pathogens, and which regulate phytoalexin accumulation (Jeandet et al. 2013, Li et al. 2019, Yang et al. 2019). Also, the integrator of the ethylene and jasmonate pathways, ERF1 acts as a substrate of MPK3/MPK6, which phosphorylate ERF1 to increase its transactivation activity and therefore further cooperate with the ethylene/jasmonate pathways to induce camalexin biosynthesis (Zhou et al. 2022). This suggests that the expression of *PepSTC* and *PepCYP* involved in phytoalexin biosynthesis was synergistically activated by jasmonate and ethylene signaling pathways.

Infection of pepper leaves by *C. coccodes* at the 8-leaf stage caused resistant lesions 96 h after inoculation (Hong et al. 2001). *CAZFP1* and *CaAMP1* transcripts were rapidly and strongly expressed during the incompatible interactions, which suggests that the genes may be involved in defense responses (Kim et al. 2004, Lee et al. 2008). Similarly, our data showed that the expression of *PepSTC* and *PepCYP* was induced early and strongly by the inoculation of *C. coccodes* in pepper leaves. The result indicates that *PepSTC* and *PepCYP* are involved in the defense response against *C. coccodes*.

Next, I confirmed to be successfully expressed *PepSTC* and *PepCYP* in *N. benthamiana* (Figure 6), and determined to be localized PepSTC and PepCYP to the cytoplasm and endoplasmic reticulum, respectively (Figure 7, 8). In the cytosol, sesquiterpenes are synthesized from isoprenoid precursors produced by the mevalonate pathway (Cankar et al. 2015). Cytochrome p450 are anchored on the outer surface of the endoplasmic reticulum (ER) by amino-terminal hydrophobic anchors (Werck-Reichhart et al. 2000). Our data show that PepSTC and PepCYP proteins were stably expressed in transgenic *N. benthamiana.* Thus, I expected to perform the functions of *PepSTC* and *PepCYP* in *N. benthamiana*

I compared extracts from transiently expressed *N. benthamiana* (*eGFP*, *PepSTC*, *PepCYP*, *PepSTC+PepCYP*) and confirmed the accumulation of phytoalexin only co-expression of *PepSTC* and *PepCYP* (Figure 10, 11). This result suggests the induction of phytoalexin accumulation by the interaction of *PepSTC* and *PepCYP*. Phytoalexin inhibited germination and appressorium formation of *C. cocodes* (Figure 12) and the hyphae growth of *C. coccodes* and lesion diameter were suppressed in transgenic *N. benthamiana* with *PepSTC* and *PepCYP* (Figure 13). In a recent study, Camalexin production was also decreased in response to bacterial or fungal infection in *Arabidopsis* (Jeandet et al. 2013). This result indicates that the phytoalexin is important for host defense against *C. coccodes* during the initial phases of infection and the accumulation of phytoalexin could enhance resistance against anthracnose fungal.

In conclusion, this study showed that the accumulation of phytoalexin was induced by the interaction of *PepSTC* and *PepCYP*, which enhances disease resistance of agianst anthracnose fungal. Furthermore, it remains to be characterized the phytoalexin to understand defense mechanism against fungal infection in pepper leaves.

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