FUNCTIONAL CHARACTERIZATION OF PUTATIVE EFFECTOR GENES OF BASIL DOWNY MILDEW PATHOGEN *PERONOSPORA BELBAHRII*

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAIʻI AT MĀNOA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

TROPICAL PLANT PATHOLOGY

MAY 2017

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Keywords: *Peronospora belbahrii,* pathogen virulence, RXLR effector, HIGS

ACKNOWLEDGEMENTS

The accomplishment of the project and thesis relied on the assistance from different individuals and institutes. Firstly, I would like to thank my supervisor Dr. Miaoying Tian for her help and guidance during the whole process. She was always there whenever I encountered any obstacle in my research progress or got stuck in writing my thesis. She was not only an advisor in research and study but also a mentor in my life. I would also like to give my thanks to my committee members, Dr. John Hu and Dr. Brent Sipes. They gave me many instructions in writing as well as conducting research. I would also thank Dr. Joe Win in Sainsbury Laboratory at United Kingdom for *in silico* identification of the *Peronospora belbahrii* effectors, and Dr. Dongliang Wu who is currently in Department of Business, Economical Development and Tourism at the State of Hawaii for analyzing the gene expression pattern using *Peronospora belbahrii*-infected basil transcriptomic data. Finally, I would acknowledge the Department of Plant and Environmental Protection Sciences, College of Tropical Agriculture and Human Resources for providing the platform and supporting my research.

ABSTRACT

Peronospora belbahrii, the causal agent of the devastating downy mildew disease on basil, is an obligate biotrophic oomycete. Similar to other oomycete pathogens, *P. belbahrii* is believed to secrete effectors to facilitate host colonization. To this end, we did the functional characterization of 10 *P. belbahrii* effector candidate genes, which encode predicted secreted proteins with a translocation motif RXLR (or RXLR-EER) and/or nuclear localization signals (NLS). First, we determined their gene expression patterns during infection using reverse transcription quantitative PCR (RT-qPCR). Five genes were induced during infection and the functionality of their predicted signal peptides was confirmed using a yeast invertase secretion system, suggesting that these genes likely encode *bona fide* effectors that play significant roles in manipulating host cellular processes to cause disease. Their roles in pathogenicity are currently being tested through overexpression and host-induced gene silencing (HIGS). To facilitate the genetic analysis of these and additional effector candidate genes, we developed a transient expression system in basil for utilizing overexpression and HIGS in a transient manner. To better analyze the effect resulted from the *in planta* expression of overexpression and HIGS construct of PbEC2 on pathogen growth, a quantitative PCR approach was developed to quantify the pathogen biomass. In addition, we generated transgenic basil to express double-stranded RNAs of one selected effector gene to determine its function in pathogenicity using HIGS. This study is expected to facilitate the understanding of *P. belbahrii* pathogenesis and help develop tools to control this pathogen.

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LIST OF ABBREVIATION

ADH	Alcohol dehydrogenase
ASGPB	Advanced Studies in Genomics, Proteomics, and Bioinformatics facility
bp	Base pair
Cb	Carbenicillin
cDNA	Complementary DNA synthesized from mRNA
ddH_2O	Double-distilled water
DMSO	Dimethyl sulfoxide
dpi	Days postinoculation
dsRNA	Double-stranded RNAs
EDTA	Ethylenediaminetetraacetic acid
gDNA	Genomic DNA
GUS	β-glucuronidase
HA	Human influenza hemagglutinin
HIGS	Host-induced gene silencing
hr	hour
IN	Agrobacteria inoculation medium
ITS	Internal transcribed spacer
LB	Luria-Bertani
MAMPs or PAMPs	Microbial- or pathogen- associated molecular patterns
min	minute
MS	Murashige and Skoog salt
NAA	1-Naphthaleneacetic acid
NB-LRR	Nucleotide binding and leucine-rich repeat
NLS	Nuclear localization signal
OD_{600}	Optical density measured at $\lambda = 600nm$
PRRs	Pattern recognition receptors
RI	Root induction medium
Rif	Rifampicin
rpm	Revolutions per minute
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
RXLR or/ dEER	Arg-X-Leu-Arg or/ Asp-Glu-Glu-Arg
SI	Callus and shoot induction medium
SDS	Sodium dodecyl sulfate
sec	Second
SOB medium	Super Optimal Broth medium
TDZ	Thidiazuron
Tris	Tris (hydroxymethyl) aminomethane
TTC	2,3,5-triphenyltertrazolium chloride
UV	Ultra violet

CHAPTER 1
INTRODUCTION

1. Basil and basil downy mildew

Basil, the common name of members of *Ocimum* genus in Lamiaceae family, is one of the most popular culinary herbs grown in North America with an annual production of approximately 4,400 hectares (Wyenandt et al., 2015) and it mainly serves as a source of edible ornamental herb, as well as essential oils, aroma compounds and anthocyanins (Simon et al., 1999; Wyenandt et al., 2015). In the state of Hawaii, basil is one of the top 20 commodities (Statistics of Hawaiian Agriculture 2011, HDOA/USDA). Among over 30 species within the genus, sweet basil (*Ocimum basilicum*) is the most commercially important species (Simon et al., 1999).

Downy mildew on *O. basilicum*, caused by the obligate biotrophic oomycete pathogen *Peronospora belbahrii*, is severely challenging the basil industry in the world (Wyenandt et al., 2010). Similar to other downy mildew pathogens, on symptomatic plants, dark purplish-brown sporangia of *P. belbahrii* are observed on the abaxial surface of leaves along with yellowing which resembles nutrient deficiency. The disease was first reported in Uganda in 1932 and after over 60 years, it re-emerged in Switzerland and was soon observed throughout European countries. Mainly through contaminated seeds, along with infected but asymptomatic plants, basil downy mildew spread distantly into the United States. The rapid spread of the pathogen and fast development of the disease have contributed to the huge yield loss of tens of millions of dollars in the US (Wyenandt et al., 2015).

Current control of basil downy mildew mainly relies on fungicides, however, conventional and organic fungicides labeled for basil downy mildew are very limited (Wyenandt et al., 2015). Since resistance to a widely-used conventional fungicide has been reported, it is very likely that *P. belbahrii* would develop resistance to the coming or registered fungicides (Cohen et al., 2013). Most of the downy mildew resistant basil identified so far are outside of the economical species (*O. basilicum*), and moreover, the sexual incompatibility and hybrid F1 sterility have been an obstacle to introducing the resistance from other *Ocimum* species (Belbahri et al., 2005). Therefore, developing alternative control methods is necessary for this devastating disease, which requires mechanical understanding of the pathogenesis mechanisms of this pathogen.

2. Molecular basis of host resistance and pathogen virulence

Based on the "zig-zag" model of plant immune system, plants use transmembrane pattern recognition receptors (PRRs) and intracellular proteins with characteristic nucleotide binding (NB) and leucine rich repeat (LRR) domains to respond to microbial- or pathogen- associated molecular patterns (MAMPs or PAMPs) and pathogen effectors, respectively. In turn, pathogens deliver effectors to conquer the immunity, which results in diseases (Jones & Dangl, 2006). This host plant-pathogen interaction is mostly applied to obligate biotrophs or hemibiotrophs, which have the intimate relationship with living hosts (Glazebrook, 2005).

3. Effectors of plant pathogenic oomycetes

Plant pathogenic oomycetes are known to secrete effectors to facilitate parasitic colonization of their hosts (Haas et al., 2009; Baxter et al., 2010; Tian et al., 2011). The effectors of this pathogen group are classified into two groups based on their targeting sites on the host. Apoplastic effectors mainly target the pathogen-induced host extracellular enzymes, whereas cytoplasmic effectors enter and function inside the host cell (Morgan & Kamoun, 2007). Based on the studies of several oomycete avirulence proteins, a modular structure of oomycete effectors was defined (Allen et al., 2004; Shan et al., 2004; Rehmany et al., 2005). In this model, the Arg-X-Leu-Arg (RXLR) translocation motif, sometimes along with its less conserved downstream Asp-Glu-Glu-Arg (dEER) domain, is required for the entry and host cell targeting (Morgan & Kamoun, 2007; Tian et al., 2011). Several identified RXLR effectors also contain nuclear localization signals (NLS), indicating their potential nuclear functions inside host cell (Morgan & Kamoun, 2007).

4. Objectives of this study

Our recent transcriptomic study of purified *P. belbahrii* sporangia and *P. belbahrii*-infected basil at different infection time courses using mRNA-seq analyses identified hundreds of putative effector genes, among which we selected 10 genes for further characterization. These 10 genes encode putative secreted proteins with translocation motif RXLR (or RXLR-EER) and/or nuclear localization signals (NLS), and they are likely upregulated during infection compared with the dormant sporangial stage based on the transcriptomic data analyses, suggesting that these

proteins enter the host cell during infection following secretion from pathogen and therefore likely play significant roles in manipulating host cellular process to cause disease. The purpose of this study is to characterize these candidate effector genes and determine whether they play significant roles in pathogenicity.

The first objective was to determine the expression patterns of 10 candidate effector genes during infection by reverse transcription quantitative polymerase chain reaction (RT-qPCR). The second objective was to determine the functionality of the putative signal peptides of the candidate genes with upregulated expression during infection. To facilitate the genetic analysis of these and additional effector candidate genes, the third objective was to develop a transient expression system in basil through GUS histochemical assays and confirm with the expression of the effector candidate gene PbEC2. We planned to utilize this system to conduct the overexpression and host-induced gene silencing (HIGS) of effector candidate genes in a transient manner. To better analyze the effect resulted from the *in planta* expression of overexpression and HIGS constructs of effector candidate genes on pathogen growth, the fourth objective was to develop a quantitative PCR (qPCR) approach to quantify the pathogen biomass. Finally, the fifth objective was to determine the role of PbEC2 in pathogenicity. PbEC2 overexpression and HIGS assays were performed in a transient manner. In addition, we generated transgenic basil plants to express double-stranded RNAs (dsRNAs) of PbEC2 to determine its function in pathogenicity using HIGS. The completion of this research is expected to facilitate the understanding of *P. belbahrii* pathogenesis mechanisms and the development of novel tools to control this disease.

CHAPTER 2:

OBJECTIVE I: Determine gene expression patterns of candidate effector genes during infection

1. INTRODUCTION

The induced expression of effector genes during infection implicates their roles in pathogenesis. Our mRNA-seq analyses suggested that the selected 10 candidate genes are expressed and induced during infection, which needs experimental validation. In this objective, we analyzed the expression patterns of *P. belbahrii* effector candidate genes during infection using RT-qPCR.

2. MATERIALS

2.1 P. belbahrii strain

The *P. belbahrii* strain used for the project was isolated from basil plants grown in Poamoho Research Station, Hawai‘i. The infected plants were kept in moist containers overnight and the sporangia were collected by swirling the detached leaves in deionized water. To obtain a single-sporangium derived isolate, diluted sporangial suspension was spread on a thin layer of 1.5% water agar, which was then cut into small pieces. The pieces were observed under microscope to select the ones with a single sporangium. The agar pieces with a single sporangium were placed on leaves of individual basil plants. The inoculated plants were incubated in trays covered with lids to maintain approximately 100% humidity, and kept in the dark for one day before being moved to the condition with a photoperiod of 12 hrs daily, 25°C and nearly 100% humidity at night for around 7 days. The newly developed sporangia were harvested from a leaf infected with a single sporangium and then propagated by spraying the suspension of 2×10^4 sporangia/ml on basil plants. The inoculated plants were treated following the same procedure for getting single-sporangium infected plants. The pathogen was maintained by reinoculation of 4-week-old basil plants approximately once per month. To prepare inocula for plant infection, we usually kept the infected plants in the tray with lid overnight to obtain more fresh sporangia, which were then collected and used as new inocula.

2.2 Basil plants

Basil seeds were provided by Enza Zaden. The specific genotype Dolly was used for pathogen inoculation in this objective. The seeds were planted in individual pots filled with soil (SunGro Horticulture Sunshine Mix #4) and grown in growth chamber conditions with a photoperiod of 12 hrs daily, 25°C. The plants were watered with fertilizer (Miracle-Gro) following the manufacturer's instructions and watered with tap water every 2-4 days. The seedlings were ready for use after 4 weeks.

2.3 Effector candidate genes

Ten effector candidate genes used in this study are listed in **Table 2.1** and named as PbEC1 PbEC2, PbEC3, PbEC4, PbEC5, PbEC6, PbEC7, PbEC8, PbEC9, PbEC10, which stands for *P. belbahrii* Effector Candidate (#). The length of the corresponding predicted amino acid sequence, the S-score calculated for predicted signal peptide, the cleavage site of signal peptide, the expression during infection, the position of RXLR motif, and the amino acid sequence for nuclear localization signal are given for each gene.

Table 2.1. The selected putative effector candidate genes of *P. belbahrii*.

Candidates	Query length	SignalP2 HMM Score	Cleavage site	Expressed during infection	RXLR position	Real RXLR effector	Nuclear Localization Signal (NLS)
PbEC1	102	0.966	26	Yes	56	Yes	
PbEC2	257	0.997	21	Yes	40 --EER=57	Yes	
PbEC3	272	0.997	21	Yes	40 --EER=57	Yes	
PbEC4	198	0.993	21	Yes	44 --EER=60	Yes	
PbEC5	121	0.997	23	Yes	46	Yes	
PbEC6	108	0.999	20	Yes	51 --EER=68	Yes	QRRRLRR (48)
PbEC7	649	1	20	Yes	44 --EER=81	Yes	KKRKDDLLDEQ LKQKLDARKQQ K
PbEC8	174	0.995	22	Yes			RRKSLEASSADG SGSSSKKRK
PbEC9	212	0.997	31	Yes			RRPVTFRKLRIL RKL (1)
PbEC10	565	0.975	22	Yes			KKKKKKK (267)

2.4 Primers used for RT-qPCR are listed in Table 2.2 below:

Table 2.2. Primers used to determine the expression patterns of ten *P. belbahrii* effector genes by RT-qPCR.

	Primer name	Sequence	Description
1	PbEC1-F	5'-GAGCATTCACACCAACAACG-3'	Forward (-F) and reverse (-R) primer for RT-qPCR of 10 *P. belbahrii* effector candidate genes.
2	PbEC1-R	5'-CCGTAAGCATCGACAACTGA-3'	
3	PbEC2-F	5'-CGACACTAATACCGGCACAA-3'	
4	PbEC2-R	5'-GGCAGCTGGACTAGAGTTCG-3'	
5	PbEC3-F	5'-CGACACTAATACGGCACAA-3'	
6	PbEC3-R	5'-GGCAGCTGGACTAGAGTTCG-3'	
7	PbEC4-F	5'-GTGGTGCGATAGACGCTGTA-3'	
8	PbEC4-R	5'-TTGGCCTGTTTCCTTTCTGT-3'	
9	PbEC5-F	5'-GTGGCTACTGCGCTCAAATC-3'	
10	PbEC5-R	5'-ATATCGTCCGGGATGGATTC-3'	
11	PbEC6-F	5'-GCGAGGAAAGATTTCCAGTG-3'	
12	PbEC6-R	5'-TCGCGTTAATGCTTGCTCT-3'	
13	PbEC7-F	5'-GGAGACAATTGGGAGACCAC-3'	
14	PbEC7-R	5'-CTTTGCTTCCAGCTTCTGCT-3'	
15	PbEC8-F	5'-GATGATCGGGTCCGTAGATG-3'	
16	PbEC8-R	5'-ACGATGACACGAAGTTGTCG-3'	
17	PbEC9-F	5'-CACCAAAGCTTCCGTCAAAT-3'	
18	PbEC9-R	5'-CTTGTGGTCAAGGAGAAGCA-3'	
19	PbEC10-F	5'-TGCAAATGAAGCGAGAGATG-3'	
20	PbEC10-R	5'-GTGCGTTGTCTACAGCCTGA-3'	
21	Pbtubulin-F	5'-CGGAAACTGGCTGTGAACTT-3'	RT-qPCR of *P. belbahrii* β-tubulin gene for normalization.
22	Pbtubulin-R	5'-CAAAGCACGGTACTGCTGAG-3'	

3. METHODS

3.1 Preparation of P. belbahrii sporangia suspension:

Infected basil plants were kept overnight in a moist container at 25°C under photoperiod of 12 hrs. The sporangia were then collected from detached leaves by immersing in distilled water. The concentration of sporangia was determined using a hemocytometer under a light microscope.

3.2 Basil inoculation and sample collection

Four-week-old Dolly, a highly susceptible genotype of sweet basil, was inoculated with the sporangial suspension (2×10^4 sporangia/ml) by spraying. The inoculated plants were kept in the dark under 100% humidity for first 24 hrs, and then under a condition with a photoperiod of 12 hrs, 25°C, ambient humidity during the day and nearly 100% humidity at night for around 7 days. Six infected whole leaves from three plants were collected at each time point from day 1 to day 5,

along with non-inoculated basil (day 0) and purified *P. belbahrii* sporangia, and frozen in liquid nitrogen.

3.3 RNA extraction and cDNA synthesis

Infected leaf samples were ground in liquid nitrogen using mortar and pestle. Total RNAs were extracted from about 100mg tissue powder using QIAGEN RNeasy Plant Mini Kit by following the instruction of the manufacturer. For RNA isolation from *P. belbahrii* sporangia, the sporangia were collected into 2 ml tubes with screw caps, to which 400µl of glass beads (425-600µm) and 450µl of buffer RLT (containing β-mercaptoethanol of 1µl/ml) (QIAGEN RNeasy Plant Mini Kit) were added. The sporangia were subjected to homogenization in high-speed bead beater (MP Biomedicals, FastPrep-24 Instrument). The 250µl of supernatant was then transferred into 1.5ml clean Eppendorf tubes after 1-min centrifugation at full speed. The pellet fractions were resuspended with another 200µl of RLT buffer containing β-mercaptoethanol, and the supernatant was collected after centrifugation. A total of 400µl of supernatant in each tube was collected. The following steps of RNA isolation were conducted following the manufacturer's instructions. All the RNA samples were treated with Ambion DNA-free kit to remove any genomic DNA. cDNA synthesis was performed using the Invitrogen SuperScript II Reverse Transcription Kit.

3.4 RT-qPCR

Primers for RT-qPCR were designed to amplify approximately 100 to120 base pairs for 10 effector candidate genes and *P. belbahrii* β-tubulin gene. The 20µl of PCR reaction was set up as follows: 1X SYBR Green mix, 250µM specific primers, 5µl of 1:30 diluted cDNA (cDNA was synthesized from 1µg total RNA in a 20µl of reaction). Programs were run at BioRad iCycler machine following the initial heating at 95°C for 30 s, then 40 thermal cycles as: denaturation at 95°C for 10 s, annealing and elongation at 60°C for 60 s. The final melting was performed by heating to 95°C, cooling down to 65°C and raising back to 95°C at 0.1°C s^{-1}. Using constitutively expressed *P. belbahrii* β-tubulin gene as an internal control as well as compared with the gene expression in pure sporangia, the relative expression level for each candidate gene (PbEC) was determined by calculation of the fold change ($2^{-\Delta\Delta Ct}$) and the formulas used were as follows (Livak & Schmittgen, 2001):

Equation 1: $\Delta Ct = Ct_{PbEC} - Ct_{\beta\text{-tubulin}}$

ΔCt was calculated for all samples, including infected basil at different time points and pure sporangia.

Equation 2: $\Delta\Delta Ct$ = ΔCt (basil samples during infection time course)- ΔCt (sporangia)

Equation 3: Fold change = $2^{-\Delta\Delta Ct}$

The fold change represents the relative expression level of the effector candidate gene at each time point during infection with the expression level in pure sporangia as 1.

Three technical replicates were set up for each biological sample and the relative expression level was calculated as the mean of fold change from three technical replicates. The standard deviation for 3 technical replicates was calculated using the formula (while the n represents the total numbers of replicates, x is the Ct value for each replicate, $\bar{x}$ represents the mean of Ct value from 3 replicates)

$$s = \sqrt{\frac{\sum (x - \bar{x})^2}{n - 1}}$$

4. RESULTS

The expression patterns of 10 candidate genes based on RT-qPCR results were generated. Relative to the expression in *P. belbahrii* sporangia, 5 candidate genes including PbEC1, PbEC2, PbEC5, PbEC9, and PbEC10, were induced during infection on plants (**Fig. 2.1**). Among them, PbEC1 and PbEC2 were the most highly induced during infection; PbEC5 and PbEC10 were also strongly induced (**Fig. 2.1**). Compared with the above 4 genes, the expression of PbEC9 during infection was slightly induced (**Fig. 2.1**). However, PbEC9 might still be a very interesting candidate to further pursue as it contains both a putative signal peptide and a nuclear localization signal (**Table 2.1**), an indicator of a *bona fide* effector protein. In total, according to their expression profile, 5 effector candidate genes that were upregulated during infection were subjected to further functional validation of their signal peptides.

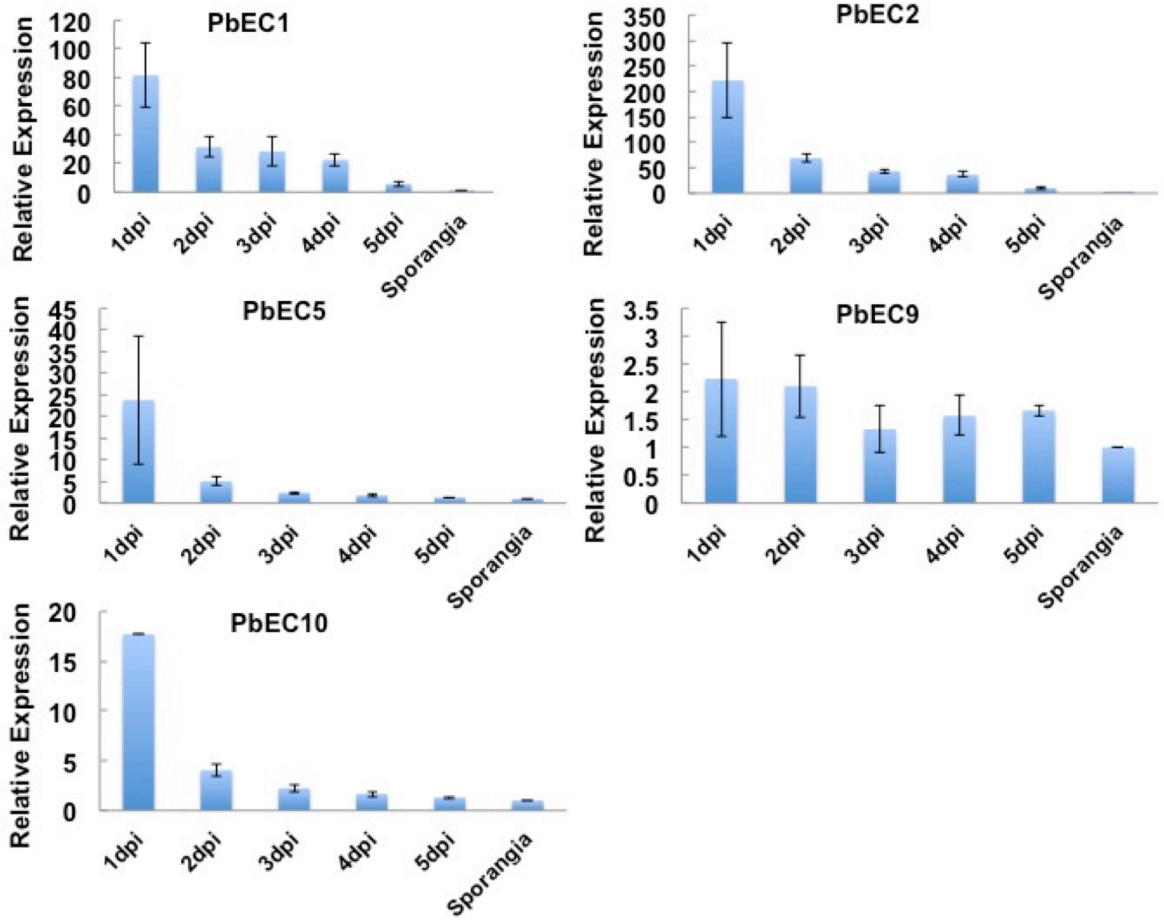


Figure 2.1. Expression patterns of 5 *P. belbahrii* putative effector genes.
Expression of PbEC1, PbEC2, PbEC5, PbEC9 and PbEC10 during infection at 1, 2, 3, 4, 5 days postinoculation (dpi). The expression level is normalized using constitutively expressed *P. belbahrii* β-tubulin gene and determined by fold change relative to *P. belbahrii* sporangia. The error bars represent ± standard deviations from three technical replicates.

CHAPTER 3

OBJECTIVE II: Validate the Functionality of the Predicted Signal Peptides of Candidate Effector Genes

1. INTRODUCTION

Oomycete cytoplasmic effectors are known to enter and function inside host cell after being secreted out of pathogen (Morgan & Kamoun, 2007), therefore validating putative signal sequence is necessary for further confirmation of effector candidate genes. To pursue the functional analysis of predicted signal peptides, the yeast secretion assays using signal sequence trap vector described by Jacobs and his associates (1997) were performed.

In nature, yeast *Saccharomyces cerevisiae* uses monosaccharide or converts polysaccharide into monosaccharide (glucose and fructose) through the enzyme activity of invertase which is encoded by SUC2 gene (Carlson et al., 1983). The yeast plasmid pSUC2T7M13ORI (pSUC2) (**Fig. 3.1**) was designed to carry with mature SUC2 lacking both its start codon and signal peptide, which leads to its defect in translation and secretion of invertase. The yeast strain YTK12 was mutated to be SUC2 defected. When a functional signal peptide was cloned into the cloning site upstream of the coding sequence of mature SUC2, the resultant functional invertase will be secreted and therefore rescue the growth of YTK12 on media using polysaccharide as sole source of carbon (Jacobs et al., 1997).

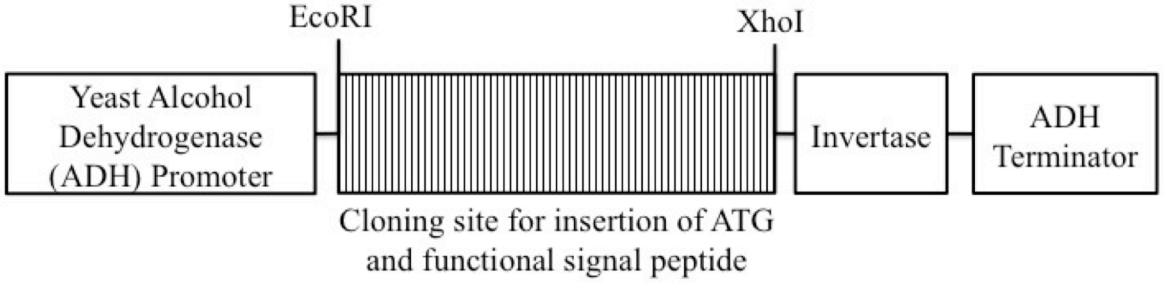


Figure 3.1. Map of cloning site of pSUC2T7M13ORI signal trap vector (modified from Jacobs et al., 1997).

2. MATERIALS

2.1 Primers used in this objective are listed in Table 3.1 below.

Table 3.1. Primers used for cloning of putative signal peptide-encoding sequences from PbEC1, PbEC2, PbEC9 and PbEC10. Restriction enzyme sites are underlined. Gene-specific sequences are in lowercase.

	Primer Name	Sequence	Description
1	PbEC1sp-FEcoR	5'-GCGTGAATTCatgcaactttcaagtatcattgcgc-3'	Cloning Signal Peptide of PbEC1 into pSUC2T7M13ORI
2	PbEC1sp-RXho	5'-GCGCTCGAGggcgtcagtgggcaaagtagt-3'	
3	PbEC2sp-FEcoR	5'-GCGGAATTCatgcgtcttgtggtgtggtt-3'	Cloning Signal Peptide of PbEC2 into pSUC2T7M13ORI
4	PbEC2sp-RXho	5'-GCGCTCGAGcgataacccattaccttgcagt-3'	
5	PbEC9sp-FEcoR	5'-GCGGAATTCatgcgacgtcctgtcacatttc-3'	Cloning Signal Peptide of PbEC9 into pSUC2T7M13ORI
6	PbEC9sp-RXho	5'-GCGCTCGAGaccaacagcatctgatggtaatagc-3'	
7	PbEC10sp-FEcoR	5'-GCGGAATTCatgttaaaactactaacgttctttc-3'	Cloning Signal Peptide of PbEC10 into pSUC2T7M13ORI
8	PbEC10sp-RXho	5'-GCGCTCGAGtcctttcagtgacaaatgcatc-3'	
9	pSUC2-F1	5'-atacggccttccttccagttacttg -3'	For sequencing the insert and colony PCR of pSUC2T7M13ORI

2.2 Plasmids used in this objective are listed in Table 3.2 below.

Table 3.2. Plasmids used to validate the functionality of predicted signal peptides of *P. belbahrii* candidate effectors.

	Plasmid	Reference	Selection marker	Description
1	pSUC2T7M13ORI (pSUC2)	Jacobs et al., 1997	Carbenicillin (Cb)	Plasmid for cloning signal peptide (SP) encoding sequences
2	pSUC2-PbEC1-SP	This study	Cb	Yeast plasmid pSUC2 containing PbEC1-SP
3	pSUC2-PbEC2-SP	This study	Cb	Yeast plasmid pSUC2 containing PbEC2-SP
4	pSUC2-PbEC9-SP	This study	Cb	Yeast plasmid pSUC2 containing PbEC9-SP
5	pSUC2-PbEC10-SP	This study	Cb	Yeast plasmid pSUC2 containing PbEC10-SP
6	pSUC2-PcQNE-SP2	Tian et al., 2011	Cb	Yeast plasmid pSUC2 containing the functional signal peptide from *Pseudoperonospora cubensis* effector

2.3 Media used in this objective are listed in Table 3.3 below.

Table 3.3. Media used for growth of yeast strain YTK12 with or without transformed yeast plasmid pSUC2.

	Name	Composition	Purpose
1	YPD medium	1% yeast extract, 2% peptone, 2% dextrose (solid medium with additional 2% agar)	Complete medium for growth of all YTK12 yeast strain
2	CMD-W medium	0.67% yeast nitrogen base without amino acids, 0.075% tryptophan dropout supplements, 0.1% glucose, 2% sucrose (solid with additional 2% agar)	Medium for growth of YTK12 yeast strain transformed with pSUC2 plasmid
3	YPRAA medium	1% yeast extract, 2% peptone, 2% raffinose and 1 µg/ml antimycin A (solid with additional 2% agar)	Selection medium for growth of YTK12 yeast strain transformed with recombinant pSUC2 plasmid containing functional signal peptide

2.4 Yeast strain and bacteria strains

Competent cells of yeast strain YTK 12 (Jacobs et al., 1997; Oh et al., 2009) used in this objective for the transformation of yeast plasmids were made following the manufacturer's instructions (Frozen EZ Yeast Transformation Kit, ZYMO RESEARCH) and stored as 100µl aliquots at -80°C. Chemical competent cells of *Escherichia coli* strain DH5α were made according to the protocol described by Green and Sambrook (2012) and stored as 100µl aliquots at -80°C.

3. METHODS

3.1 Cloning of putative signal peptides from 4 effector candidate genes into pSUC2 plasmid

3.1.1 Amplification of DNA fragments encoding predicted signal peptides

Putative signal peptides from 4 effector candidate genes (PbEC1, PbEC2, PbEC9, and PbEC10) were amplified using gene specific primers listed in **Table 3.1**. *P. belbahrii* genomic DNA (gDNA) was used as templates for PCR amplification. The reagents in the 50µl of PCR reaction were as follows: 140ng *P. belbahrii* gDNA, 0.2mM dNTPs, 1X HF buffer, 0.4µM specific primers, and 1 unit of Phusion High-Fidelity DNA polymerase (NEB). PCR conditions started from an initial denaturation of 30 sec at 98°C, followed by 35 cycles of thermocycling: 15 sec at 98°C for denaturation, 15 sec at corresponding temperature for annealing (the annealing temperature suggested by NEB T_m calculator for PbEC1, PbEC2, PbEC9, and PbEC10 were 70°C, 63°C, 68°C, and 61°C, respectively), 30 sec at 72°C for elongation. The final step of elongation took place at 72°C for 10 min.

The resulted PCR products were purified using QIAGEN QIAquick PCR Purification Kit following the manufacturer's instructions.

3.1.2 Digestion of signal peptides and pSUC2 plasmid

The double digestion was conducted for the amplified signal peptides and pSUC2 plasmid. The reagents added into the 50µl of digestion reaction were: 30µl of PCR product or plasmid pSUC2, 1X NEB Buffer 3.1, 10 units EcoRI (NEB), 10 units XhoI (NEB), and ddH_2O up to 50µl. The reactions then were incubated at 37°C for 3 to 4 hrs.

3.1.3 Visualization and extraction of digested products

Digestion products were resolved on 1% agarose gel by electrophoresis under 120 V and 500 mA for 35 min. The digested plasmid was excised from the gel and extracted using QIAquick Gel Extraction Kit. The digested signal peptides were purified using QIAGEN QIAquick PCR Purification Kit.

3.1.4 Ligation of signal peptides into linearized pSUC2 plasmid

The ligation was performed in a 15µl of reaction with the ingredients added as follows: double digested plasmid and signal peptides, 400 units of T4 DNA ligase (NEB), 1X ligase buffer. The reactions were incubated overnight at room temperature and stored at 4°C.

3.1.5 Transformation through heat shock into E. coli strain DH5α

The 100µl aliquot of *E. coli* DH5α competent cells from -80°C was thawed on ice for 15 min. Half of the ligation reactions were added into the competent cells and incubated on ice for 30 min. The Eppendorf tubes with ligation reaction and *E. coli* DH5α were incubated in water bath at 42°C for 45 sec and then returned to ice for 2 min. After the incubation, 1ml of LB medium was added to the tubes. The tubes were shaken at 250 rpm for 1 hr at 37°C. 100µl of the resulting culture were spread on LB plates containing 50µg/ml Carbenicillin (Cb) and grown overnight at 37°C. The single colonies appeared next morning were streaked on new LB plates containing 50µg/ml Cb, and subjected to colony PCR.

3.1.6 Colony PCR and sequencing

The colony PCR was conducted using pSUC2-F1 and gene-specific reverse primers (**Table 3.1**). The 25µl of PCR reaction contained the following ingredients: 200µM dNTPs, 1X Taq buffer, 1.5mM $MgCl_2$, 0.5 µM specific primers and 1 unit of Taq DNA polymerase (Lambda biotech). A small amount of cells were scraped from a single colony using a pipette tip and mixed to the PCR reaction solution. PCR conditions started from an initial denaturation of 2 min at 94°C, followed by 35 cycles of thermocycling: 15 sec at 94°C for denaturation, 30 sec at 54°C for annealing, and 1 min at 72°C for elongation. The final step of elongation took place at 72°C for 5 min. PCR products were visualized on a 1% agarose gel by electrophoresis under 120 V and 500 mA for 35 min. The colonies with expected size of PCR product were inoculated into LB liquid medium containing 50µg/ml Cb and incubated at 250 rpm, 37°C for overnight. The DNA of recombinant plasmids then was extracted using QIAGEN QIAprep Spin Miniprep Kit. The generated 200-300ng of DNA and 3.2pmol of pSUC2-F1 primer were mixed and sent (ASGPB facility, UH Manoa) for sequencing. The plasmids with the right inserts were chosen to make DMSO stock and stored at -80°C.

3.2 Yeast transformation

The resulted plasmids cloned with specific signal peptides were extracted using QIAGEN QIAprep Spin Miniprep Kit and transformed into 50µl of YTK12 yeast competent cells using Frozen EZ Yeast Transformation Kit (ZYMO RESEARCH). A 50µl aliquot of transformation reaction was spread on CMD-W plate and incubated at 30°C for 3 days.

3.3 Functional analysis of signal peptides using yeast secretion system

3.3.1 Yeast medium growth assay

The transformants were selected on CMD-W (minus Trp) media. The single colonies of transformed YTK12, along with YTK12, YTK12 carrying empty vector pSUC2, YTK12 transformed with the pSUC2 carrying a previously identified functional signal peptide PcQNE-SP2 (Tian et al., 2011) as controls, were streaked onto YPRAA plates, CMD-W plates, and YPD plates (serving as viability controls).

3.3.2 TTC test

The functionality of signal peptides was also tested by an invertase enzymatic activity assay through TTC (2,3,5-triphenyltertrazolium chloride) test as described by Oh et al., 2009. In detail, the pSUC2 recombinant plasmid and pSUC2 empty vector transformed YTK12 colonies were inoculated into 5ml of CMD-W liquid medium, while YTK12 without transformed plasmid was inoculated into 5ml of YPD medium. The inoculated media were incubated at 30°C, 250 rpm for around 20 hrs. The yeast cells were pelleted by centrifugation at 4000 rpm for 5 min (Eppendorf Centrifuge 5810). The collected pellets were washed with ddH_2O twice and resuspended in 5 ml ddH_2O. The suspensions were further diluted in ddH_2O to obtain a final OD_{600} of 0.6. 1ml of the resulted suspensions were added into 5ml of 5% sucrose, following by adding 1% TTC solution into the suspension and incubated at 35°C of water bath for 35 min. 100µl of 10N NaOH was then added into the suspensions and incubated at room temperature for another 5 min.

4. RESULTS

The putative signal peptides from PbEC1, PbEC2, PbEC9 and PbEC10 were validated using the yeast invertase secretion system. The predicted signal peptides were amplified and fused in frame with the mature sequence of yeast invertase SUC2 in pSUC2 vector. The recombinant plasmids were then transformed into YTK12. The functional signal peptide will enable the secretion of invertase (Jacobs et al., 1997) for converting sucrose or raffinose into monosaccharide (glucose and fructose) (Carlson et al., 1983).

In the yeast growth assay, each of three plates containing YPD, CMD-W, or YPRAA media was divided into 4 parts for streaking 4 different yeast strains. YPD and CMD-W media were used to confirm the viability and authenticity of these strains. YPD medium is a complete medium for yeast growth and was used as a viability control. All strains grew equally well on YPD media, suggesting that they were all viable for the assays (**Fig. 3.2 A, Fig. 3.3 A, Fig. 3.4 A, Fig. 3.5 A)**. CMD-W is a complete minimal medium without tryptophan (Trp) and was used to confirm whether the strains containing pSUC2 or its derived plasmids as they carry TRP1 gene for synthesizing Trp, an essential amino acid for yeast growth. As expected, all YTK12 strains containing pSUC2 or its derived plasmids grew on CMD-W media while YTK12 itself did not

grow (**Fig. 3.2 A, Fig. 3.3 A, Fig. 3.4 A, Fig. 3.5 A).** Both YPD and CMD-W contain glucose, the monosaccharide required for the growth of YTK12 strains. In contrast, the only carbon source in YPRAA media is raffinose, a trisaccharide that cannot be directly used by invertase-deficient YTK12 yeast strains. However, when YTK12 carries pSUC2 expressing a functional signal peptide, it will secrete a functional invertase to metabolize raffinose to monosaccharide to enable its growth. Indeed, similar to the positive control - YTK12 with pSUC2 carrying a characterized functional signal peptide from *Pseudoperonospora cubensis* effector PcQNE (PcQNE-SP2), all the resultant strains with pSUC2 containing signal peptide from PbEC1, PbEC2, PbEC9 or PbEC10 (PbEC1-SP, PbEC2-SP, PbEC9-SP, or PbEC10-SP) grew on YPRAA plates while YTK12 and YTK12 carrying mature SUC2 failed to grow (**Fig. 3.2 A, Fig. 3.3 A, Fig. 3.4 A, Fig. 3.5 A**), indicating the tested signal peptides are functional.

The functionality of the signal peptides described above was also confirmed through TTC test. In this test, the secreted invertase would lead to the hydrolysis of sucrose into glucose and fructose, which can positively react with non-colored TTC to produce the red colored triphenylformazan (TRF). The red color was detected for all the tested signal peptides (**Fig. 3.2 B, Fig. 3.3 B, Fig. 3.4 B, Fig. 3.5 B**).

The results from both yeast secretion assays proved that PbEC1, PbEC2, PbEC9, and PbEC10 encode secreted proteins and would be further pursued for their roles in pathogenicity.

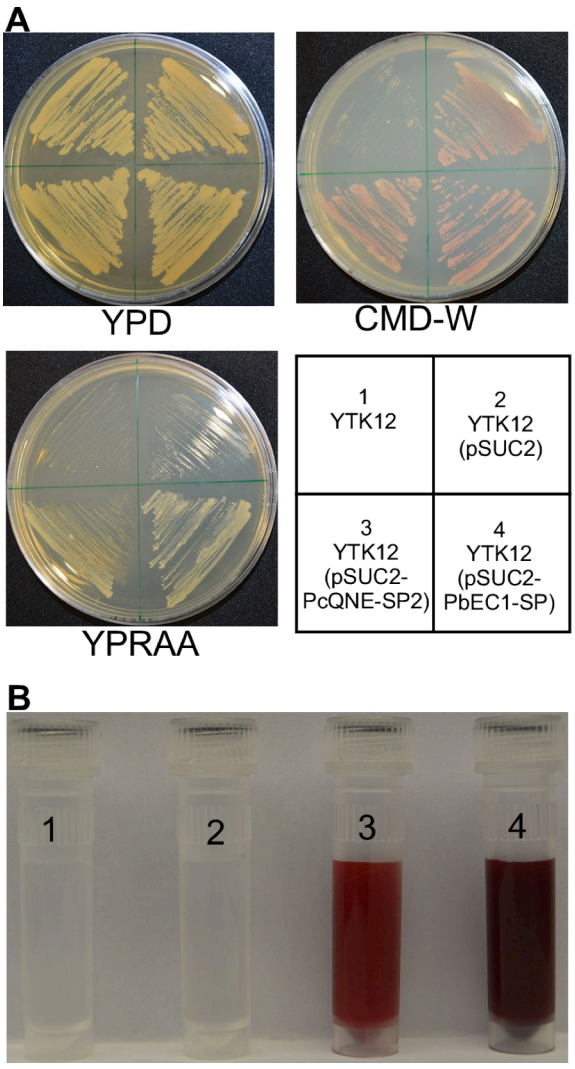


Figure 3.2. Functional analyses of signal peptide from PbEC1 (PbEC1-SP) through yeast invertase secretion assays. (**A**) Yeast medium growth assay on YPD (yeast extract – peptone – dextrose), CMD-W (without tryptophan) and YPRAA (yeast extract – peptone – raffinose - antimycin – agar) media. Similar to YTK12 with pSUC2 carrying the characterized functional PcQNE-SP2 (3) (Tian et al., 2011), YTK12 strain with pSUC2 fused with PbEC1-SP (4) can grow on YPRAA media, whereas YTK12 (1) and YTK12 carrying pSUC2 empty vector (2) were unable to grow on YPRAA media. (**B**) Yeast invertase enzymatic activity through TTC (2,3,5-triphenyltertrazolium chloride) test. The red color was observed on tested signal peptide of PbEC1. The numbers were labeled as A.

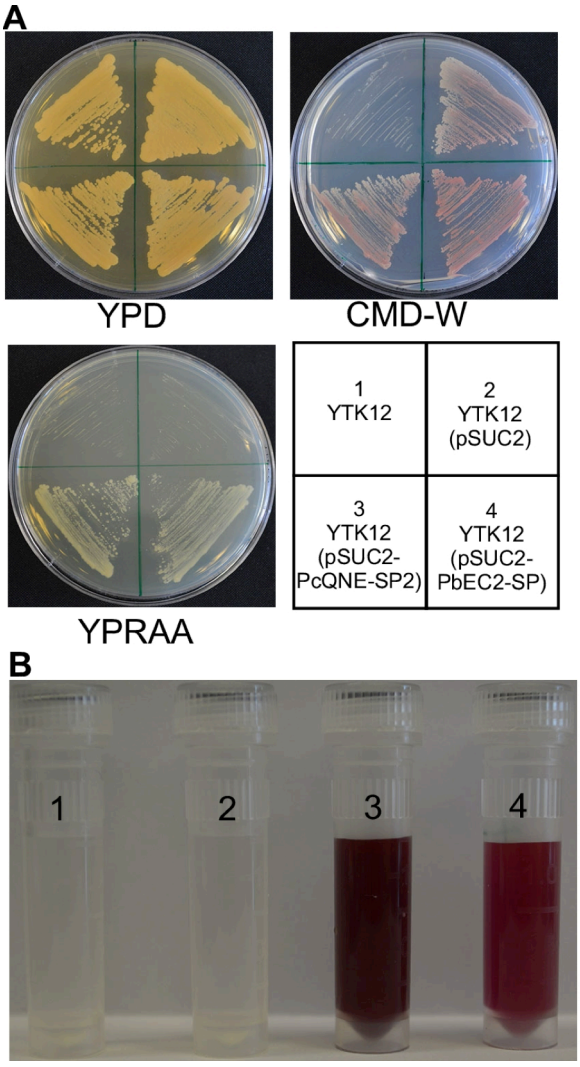


Figure 3.3. Functional analyses of signal peptide from PbEC2 (PbEC2-SP) through yeast invertase secretion assays.
(**A**) Yeast medium growth assay on YPD (yeast extract – peptone – dextrose), CMD-W (without tryptophan) and YPRAA (yeast extract – peptone – raffinose - antimycin – agar) media. Similar to YTK12 with pSUC2 carrying the characterized functional PcQNE-SP2 (3) (Tian et al., 2011), YTK12 strain with pSUC2 fused with PbEC2-SP (4) can grow on YPRAA media, whereas YTK12 (1) and YTK12 carrying pSUC2 empty vector (2) were unable to grow on YPRAA media. (**B**) Yeast invertase enzymatic activity through TTC (2,3,5-triphenyltertrazolium chloride) test. The red color was observed on tested signal peptide of PbEC2. The numbers were labeled as A.

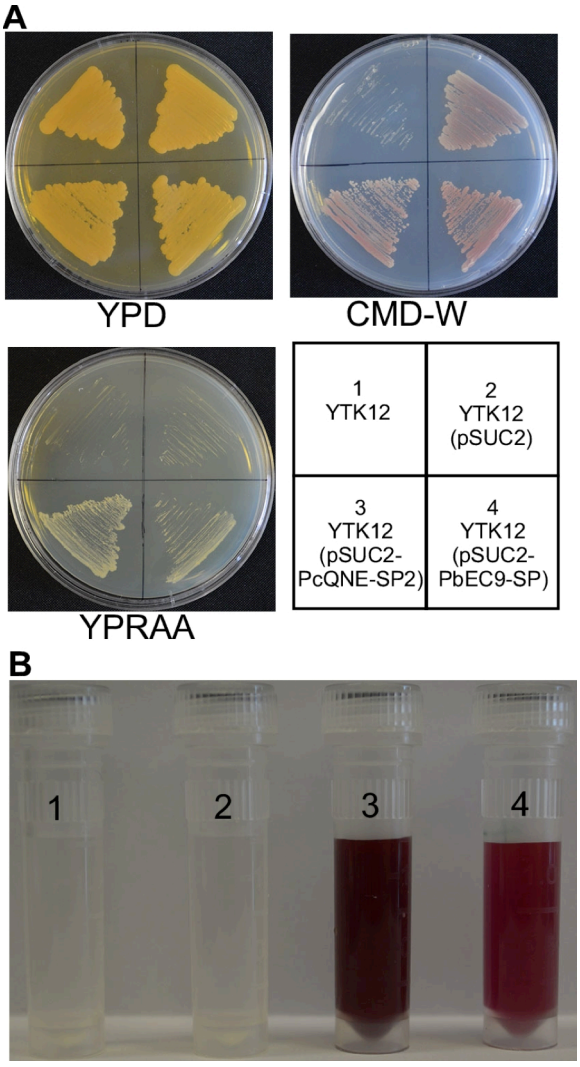


Figure 3.4. Functional analyses of signal peptide from PbEC9 (PbEC9-SP) through yeast invertase secretion assays.
(**A**) Yeast medium growth assay on YPD (yeast extract – peptone – dextrose), CMD-W (without tryptophan) and YPRAA (yeast extract – peptone – raffinose - antimycin – agar) media. Similar to YTK12 with pSUC2 carrying the characterized functional PcQNE-SP2 (3) (Tian et al., 2011), YTK12 strain with pSUC2 fused with PbEC9-SP (4) can grow on YPRAA media, whereas YTK12 (1) and YTK12 carrying pSUC2 empty vector (2) were unable to grow on YPRAA media. (**B**) Yeast invertase enzymatic activity through TTC (2,3,5-triphenyltertrazolium chloride) test. The red color was observed on tested signal peptide of PbEC9. The numbers were labeled as A.

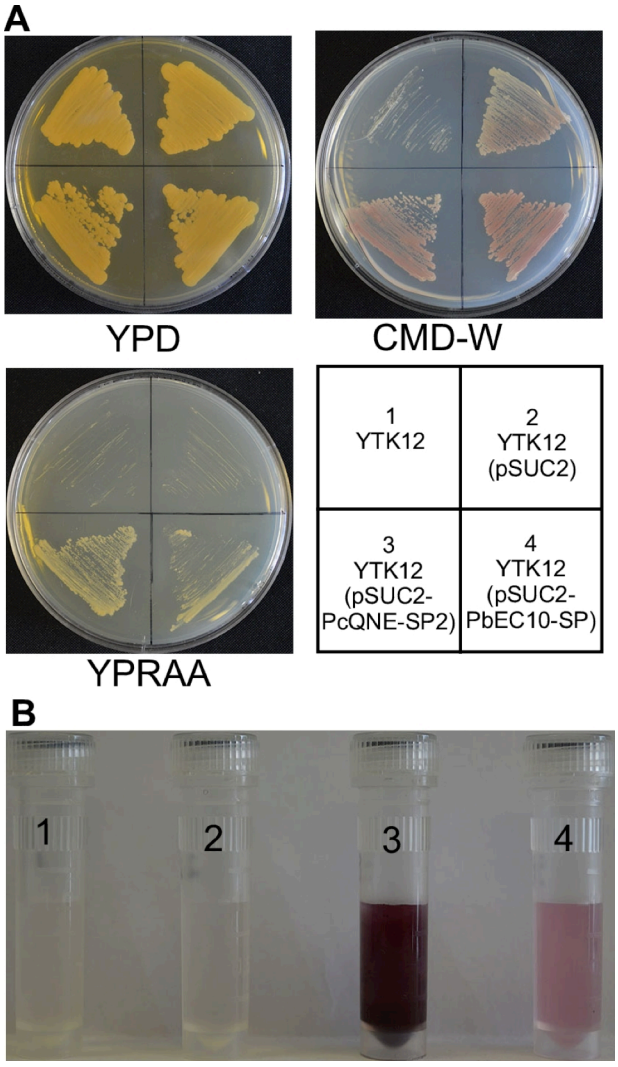


Figure 3.5. Functional analyses of signal peptide from PbEC10 (PbEC10-SP) through yeast invertase secretion assays.
(**A**) Yeast medium growth assay on YPD (yeast extract – peptone – dextrose), CMD-W (without tryptophan) and YPRAA (yeast extract – peptone – raffinose - antimycin – agar) media. Similar to YTK12 with pSUC2 carrying the characterized functional PcQNE-SP2 (3) (Tian et al., 2011), YTK12 strain with pSUC2 fused with PbEC10-SP (4) can grow on YPRAA media, whereas YTK12 (1) and YTK12 carrying pSUC2 empty vector (2) were unable to grow on YPRAA media. (**B**) Yeast invertase enzymatic activity through TTC (2,3,5-triphenyltertrazolium chloride) test. The red color was observed on tested signal peptide of PbEC10. The numbers were labeled as A.

CHAPTER 4

OBJECTIVE III: Develop transient expression system in basil

1. INTRODUCTION

P. belbahrii is an obligate biotrophic oomycete pathogen which is unable to be directly genetically modified (Wyenandt et al., 2010). Alternatively, the genetic characterization of its effector candidate genes can be processed through their expression in host plants. Compared with stably expressing the candidate genes through generating transgenic plants, transiently expressing genes using agrobacterium-mediated transformation is more economical and efficient to screen a number of candidates within a short span of time. Given that the transient expression has not been tested on basil yet, our objective was to establish a transient system in basil for further characterization of *P. belbahrii* effectors and understanding of the pathogenesis mechanism.

To develop a transient gene expression system in basil, we first used a GUS (β-glucuronidase) reporter system to detect the expression of GUS by visualizing its enzymatic activity. When the enzyme reacts with colorless substrate 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc), the blue colored products will be observed. The GUS gene has been widely used as a marker for gene expression in higher plants (Jefferson, Kavanagh, & Bevan, 1987). This transient expression system was further tested with a construct carrying PbEC2.

2. MATERIALS

2.1 Basil and Nicotiana benthamiana

Seeds for 6 basil genotypes, including Marian, Jolina, Eowyn, Genovese, Emily and Dolly, were provided by Enza Zaden. Basil seeds were planted in 3.5" square pots (McCONKEY) filled with soil mix (SunGro Horticulture Sunshine Mix #4) and placed in a growth chamber with conditions at 25°C, photoperiod of 12 hrs, for 3 to 4 weeks. Under the same growth conditions, around 20 *N. benthamiana* seeds were first grown in a 8" AZ green pot (Niu Nursery, Honolulu, HI) filled with soil mix for 2 weeks, and the 2 to 3-cm-long seedlings were then transferred into individual 5" green pots with soil for another 3 weeks. The plants were fertilized every 2 weeks and watered with tap water every 2-4 days.

2.2 Plasmids used in this objective are listed in Table 4.1.

Table 4.1. Plasmids used for transient expression of GUS and PbEC2 in basil.

	Name	Reference	Description
1	pBISN1	Narasimhulu et al., 1996	Transient expression of GUS in basil
2	pE1776	Lee et al., 2007	Plant binary vector used to construct pE1776-2HA
3	pE1776-2HA	This study	A modified plant binary vector for expressing fusion proteins at the C-termini in plants
4	pE1776-2HA-PbEC2	This study	Transient expression of PbEC2 fused with 2HA tag at the C-terminus

2.3 Primers used in this objective are listed in Table 4.2.

Table 4.2. Primers used for making the construct to express PbEC2 in basil. Restriction enzyme sites are underlined and gene-specific sequences are in lowercase.

	Name	Sequence	Description
1	2HA-F1	CTAGTtacccatacgatgttccagattacgcttacccatac gatgttccagattacgctTAAGAGCT	Cloning of 2HA tag into pE1776
2	2HA-R1	CTTAagcgtaatctggaacatcgtatgggtaagcgtaatct ggaacatcgtatgggtaA	
3	pMSP1-F1	tggataaatagccttgcttcct	Colony PCR and sequencing for pE1776-derived plasmids
4	pMSP1-R1	gagcatggtctcagcgccggct	
5	PbEC2-Fhind	gcgAAGCTTatggtgggagaccccatagcgaccacaa	Cloning of PbEC2 into pE1776-2HA
6	PbEC2-Rspe	gcgACTAGTggcgccagaggcatgaactt	

2.4 Bacterial strains

The competent cells of *Agrobacterium tumefaciens* strains EHA105 and GV3101, and *E. coli* strain DH5α were used in this objective. Competent cells of *A. tumefaciens* strains EHA105 and GV3101 used for electroporation were made according to the protocol described by Green and Sambrook (2012) and stored as 100µl aliquots at -80°C. Chemical competent cells of *E. coli* strain DH5α were made and stored as described in Chapter 3 (2.4).

2.5 Media and buffers used in the objective are listed in Table 4.3 below.

Table 4.3. Media and buffers used for transient expression and detection of GUS in basil.

	Name	Composition	Purpose
1	Infiltration/Induction media	10 mM MES pH 5.75, 10 mM magnesium chloride, 150 µM acetosyringone	Making Agrobacteria suspension for agro-infiltration on basil leaves
2	GUS staining solution	100 mM sodium phosphate at pH7.0, 1.0 mM potassium ferricyanide, 10mM EDTA, 0.1% Triton X-100 and 2.0 mM X-Gluc	Solution for visualization of GUS expression in basil leaves
3	Destaining solution	50% ethanol	Destaining the background from chlorophyll for GUS visualization

3. METHODS

3.1 Transient expression of GUS and GUS staining

3.1.1 Agro-infiltration of Agrobacterium GV3101 harboring pBISN1

Agrobacterium GV3101 with pBISN1 which contains a *gusA* (uidA) gene under a super promoter (Narasimhulu et al., 1996) was used for *A. tumefaciens*-mediated transient gene expression. 6 sweet basil genotypes (Marian, Jolina, Eowyn, Genovese, Emily, and Dolly) were used for this assay, and *N. benthamiana* whose transient expression system is well established served as a positive control.

The *A. tumefaciens* strain was retrieved from -80°C and incubated on LB agar plates containing 15µg/ml Rifampicin, 25µg/ml Gentamicin, 50µg/ml Kanamycin at 28°C for 2 days, and re-streaked onto a fresh plate the day before use. The next day, the bacteria were re-suspended in the incubation/infiltration media to a final OD_{600} of 0.4. The suspension then was induced at room temperature for 2 hrs by periodically mixing. The *A. tumefaciens*-mediated transient gene expression was performed by infiltrating the bacteria suspension into the abaxial side of the second pair of true leaves using 1 ml needleless syringe. Six plants of each genotype were infiltrated.

3.1.2 GUS staining

GUS activity was visualized through histochemical assay according to the protocol from Jefferson at 1987. For each basil genotype, 2 samples were collected at 5 day after infiltration. For each sample, total 4 basil leaf disks of 1 cm^2 (one from one leaf, 2 leaves

from each plant) were collected with a cork-borer and vacuum–infiltrated with GUS staining solution. The resultant leaf disks were then incubated in the staining solution at 37°C for overnight. The next day, the samples were immersed in destaining solution and placed in the spinning machine (RotoFlex). The destaining step was repeated until the leaf surface became bleached. The blue stains were detected and recorded by Olympus BX41 microscope.

3.2 Construction of pE1776-2HA

3.2.1 Annealing of 2HA tag

The oligonucleotides 2HA-F1 and 2HA-R1 were annealed to form 2HA tag with added digested SpeI and SacI cohesive ends. The following ingredients were added into the 20µl of reaction: 2.5 µM specific primers, 1X ligase buffer, and ddH_2O. The Eppendorf tube containing the annealing reaction was then incubated in the beaker with water of 95°C and slowly cooled down and left overnight at room temperature.

3.2.2 Digestion of pE1776

pE1776 plasmid from *E. coli* DH5α was isolated using QIAGEN QIAprep Spin Miniprep Kit following the manufacturer's instructions. The 30µl of the resultant plasmid was digested in the 50µl of reaction containing 1X NEB CutSmart Buffer, 10 units SpeI (NEB), 10 units SacI (NEB), and ddH_2O. The reactions then were incubated at 37°C for 3 to 4 hrs.

3.2.3 Visualization and purification of digested products

Digested products were resolved on 1% agarose gel by electrophoresis under 120 V and 500 mA for 35 min, and isolated from the gel with QIAquick Gel Extraction Kit. The extracted digested products were used for ligation.

3.2.4 Ligation of digested pE1776 and 2HA tag

The ligation was performed for the digested pE1776 and 2HA tag. The 15µl of reaction consisted of digested plasmid, annealed 2HA tag, 400 units of T4 DNA ligase (NEB), 1X

ligase buffer, and ddH_2O. The reaction was incubated overnight at room temperature and stored at 4°C before transformation.

3.2.5 Transformation of pE1776-2HA into E. coli DH5α through heat shock

The experiments were conducted as described in Chapter 3 (3.1.5).

3.2.6 Colony PCR and sequencing

The procedure was as described in Chapter 3 (3.1.6) using the primer pair pMSP1-F1 and pMSP1-R1.

3.3 Vector construction for transient expression of PbEC2

3.3.1 Amplification of PbEC2

PbEC2 was amplified using gene specific primers PbEC2-Fhind and PbEC2-Rspe listed in **Table 4.2**. 2μl of 1:30 diluted cDNAs of 4dpi sample generated in Chapter 2 (3.3) was used as templates for PCR amplification. The reagents in the 50μl of PCR reaction were as follows: 4dpi cDNAs, 0.2mM dNTPs, 1X HF buffer, 0.4μM specific primers, and 1 unit of Phusion High-Fidelity DNA polymerase (NEB). PCR conditions started from an initial denaturation of 30 sec at 98°C, followed by 35 cycles of thermocycling: 15 sec at 98°C for denaturation, 15 sec at 70°C for annealing, 1 min at 72°C for elongation. The final step of elongation took place at 72°C for 10 min. The resulted PCR products were purified using QIAGEN QIAquick PCR Purification Kit following the manufacturer's instructions.

3.3.2 Digestion of PbEC2 and pE1776-2HA

pE1776-2HA and PbEC2 were digested with HindIII and SpeI in 50μl of reactions containing 30μl of plasmid or PbEC2, 1X NEB Buffer 2.1, 10 units HindIII (NEB), 10 units SpeI (NEB), and ddH_2O. The reactions were incubated at 37°C for 3 to 4 hours.

3.3.3 Visualization of digested products and gel extraction

Digested products were resolved in 1% agarose gel by electrophoresis under 120 V and 500 mA for 35 min, excised from the gel and extracted using QIAquick Gel Extraction Kit.

3.3.4 Ligation of PbEC2 to linearized pE1776-2HA plasmid

The ligation was conducted by adding digested PbEC2, linearized pE1776-2HA plasmid, 400 units of T4 DNA ligase (NEB), 1X ligase buffer and ddH_2O into the 15µl of reaction, followed by incubation overnight at room temperature. The ligation products were stored at 4°C until transformation.

3.3.5 Transformation through heat shock into E. coli DH5α

The heat shock was performed as Chapter 3 (3.1.5).

3.3.6 Colony PCR and sequencing

The PCR and sequencing were conducted using the primer pair pMSP1-F1 and pMSP1-R1 according to the description in Chapter 3 (3.1.6).

3.3.7 Transformation into Agrobacterium EHA105 through electroporation

Aliquot of Agrobacterium EHA105 competent cells was taken out of -80°C and thawed on ice. The 1µl of pE1776-2HA-PbEC2 plasmid isolated using QIAGEN QIAprep Spin Miniprep Kit was mixed with 100µl of Agrobacteria and transferred into a pre-cooled electroporation cuvette (Bull-dog bio). The cuvette then was processed into electroporation in Eporator (Eppendorf). 1ml of LB was added into the cuvette immediately after electroporation and mixed and transferred to the Eppendorf tube. The tube was incubated at 28°C, 250rpm for 1.5 hr. 50µl of suspension from the tube was spread on a new LB plate containing 15µg/ml Rifampicin, 25µg/ml Gentamicin and 50µg/ml Kanamycin and incubated at 28°C for 2 days. The single colonies that appeared on the plate was re-streaked on a new LB plate with same antibiotics and confirmed by colony PCR using the primer pair pMSP1-F1 and pMSP1-R1. The one with right insert was chosen to make stock.

3.4 Transient expression of PbEC2 in basil and N. benthamiana

3.4.1 Agro-infiltration of Agrobacterium strain EHA105 containing pE1776-2HA-PbEC2

The procedure of the infiltration on 2 basil genotypes – Jolina and Emily and *N. benthamiana* was the same as described in 3.1.1. Five plants for each basil genotype and 5 leaves from one *N. benthamiana* plant were infiltrated.

3.4.2 Sample collection, RNA extraction, and cDNA synthesis

Three days after agroinfiltration, the leaf samples from Jolina, Emily (two basil genotypes) and *N. benthamiana* were collected. The subsequent procedures from RNA extraction to cDNA synthesis were conducted as Chapter 2 (3.2 and 3.3).

3.4.4 Regular PCR to amplify PbEC2

The 5µl of 1:10 diluted cDNA was added into 25µl of reaction containing 200µM dNTPs, 1X Taq buffer, 1.5mM $MgCl_2$, 0.5µM PbEC2-Fhind and PbEC2-Rspe primers, and 1 unit of Taq DNA polymerase (Lamda biotech). The amplicons of regular PCR were resolved in 1 % agarose gel by electrophoresis under 120 V and 500 mA for 35 min and observed under UV light.

4. RESULTS

4.1 Transient expression of GUS was visualized in basil

GUS expression was visualized in all the tested samples with expression level varying among different genotypes. Marian and Genovese were shown to have the highest expression level whereas GUS expression with very faint staining was observed in Eowyn and Dolly (**Fig. 4.1).**

4.2 Transient expression of PbEC2 was detected in basil

Consistent with the capacity of transient expression confirmed by GUS staining, the expression of PbEC2 was detected by RT-qPCR (data not shown) and regular RT-PCR (**Fig. 4.2**). In total, agrobacterium-mediated transient expression seems to be a feasible tool to express foreign genes in basil, which would be further utilized for genetic characterization of the candidate genes.

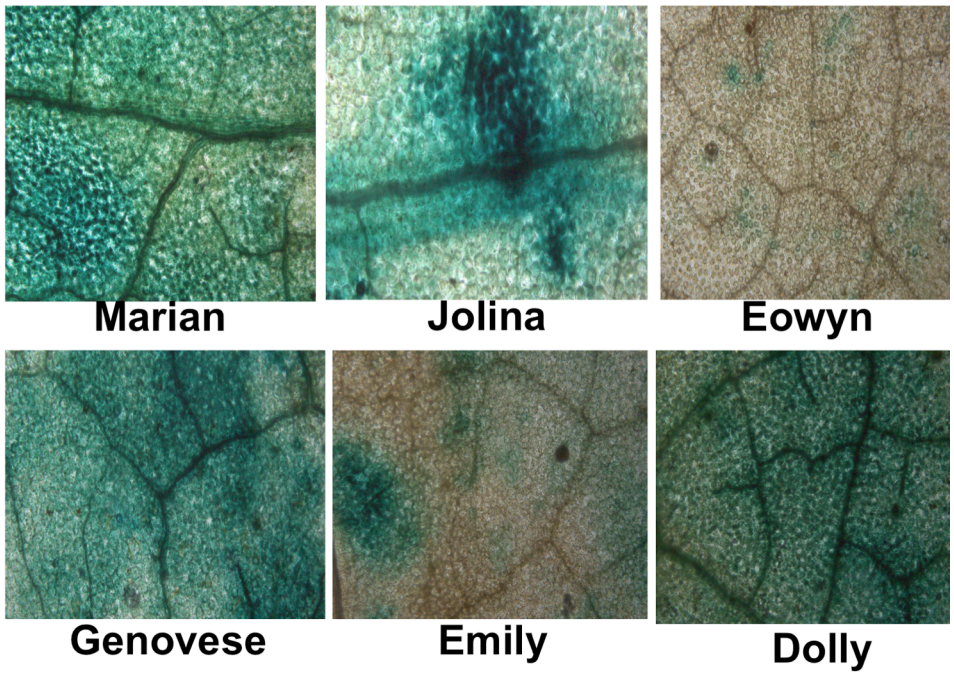


Figure 4.1. Transient expression of GUS in 6 basil genotypes. Blue stains indicate the GUS expression.

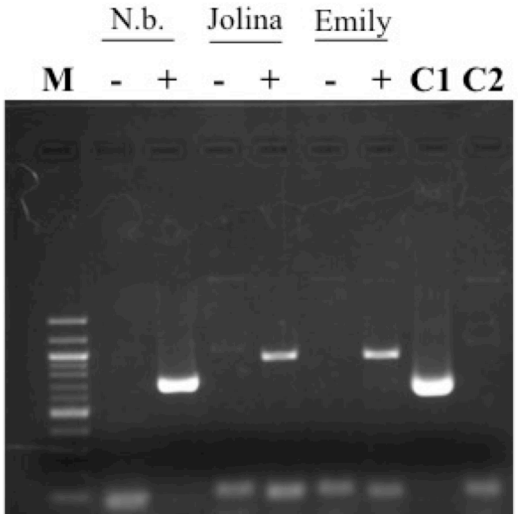


Figure 4.2. Agarose gel of PCR products of PbEC2 amplified from *Nicotiana benthamiana* (N.b.), Jolina and Emily (two basil genotypes) infiltrated with (+) or without (-) agrobacterium strain EHA105 carrying pE1776-2HA-PbEC2. C1, PCR product with the pure pE1776-2HA-PbEC2 plasmid as the template; C2, no template control.

CHAPTER 5

OBJECTIVE IV: Develop a qPCR approach to quantify *P. belbahrii* during infection

1. INTRODUCTION

Similar to other biotrophic pathogens, the roles of *P. belbahrii* effector candidate genes in pathogenicity can be identified through their *in planta* expression. This genetic identification is often assessed by the pathogenicity test where the disease level is determined by the altered pathogen virulence or plant susceptibility. The traditional means of evaluating the disease severity include scoring the visual symptoms or pathogen structures (Brouwer et al., 2003), such as measuring the lesion size (Brouwer et al., 2003) or counting the conidiophores (Anderson & McDowell, 2015). However, these conventional methods have their disadvantages, for instance, the plants with invisible symptoms may also show positive results in pathogen colonization (Hukkanen et al., 2006), the counting of spores may be affected by variability resulted from the collection methods (Anderson & McDowell, 2015). In terms of accuracy, those approaches cannot offer the precision in small changes of pathogen virulence or plant susceptibility (Anderson & McDowell, 2015). In our study, transient expression will be used to determine the role of candidate effector genes in pathogenicity. As high-level of expression only lasts a short period of time, it is essential to perform disease assays at early infection time when visual symptoms are still not detectable. Therefore, a sensitive approach that allows quantitative assessment of pathogen growth is needed.

Quantitative PCR (qPCR) has been utilized for the quantification of several downy mildew pathogens, such as *Peronospora sparsa* in *Rubus* species (Hukkanen et al., 2006) and *Hyaloperonospora arabidopsidis* in *Arabidopsis thaliana* (Anderson & McDowell, 2015). With specific primers that amplify the pathogen gene and the plant gene respectively (Hukkanen et al., 2006; Anderson & McDowell, 2015), the pathogen growth can be accurately quantified as the ratio of the amplification of the pathogen gene to the plant gene, and can be achieved even prior to the reproductive stage to detect the small variation in pathogen virulence.

Ribosomal DNA has been widely used as target genes for detection and quantification of plant pathogens (Brouwer et al., 2003; Hukkanen et al., 2006; Anderson & McDowell, 2015) due to its

specificity for pathogen and sensitivity conferred by high copy numbers. In this objective, the primers amplifying the internal transcribed spacer 2 (ITS2) region of *P. belbahrii* were used in qPCR for pathogen quantification. The primers amplifying basil β-tubulin gene were used for normalization. The specificity and efficiency of the primer sets were tested. This method is expected to be utilized in the future genetic identification assays to evaluate the effect resulted from *in planta* expression of effector candidate genes.

2. MATERIALS

2.1 Basil and P. belbahrii strain

Basil genotypes Dolly and Genovese were used and grown under the same conditions as described in Chapter 4 (2.1). The *P. belbahrii* strain used and its maintenance were described in Chapter 2 (2.1).

2.2 Buffer

The components of crude DNA extraction buffer used to extract the genomic DNA of infected basil tissue are listed in Table 5.1 below.

Table 5.1. Buffer used for extracting genomic DNA from infected basil tissues.

Name	Components	Purpose
Crude DNA extraction buffer	200mM Tris (tris (hydroxymethyl) aminomethane), pH 7.5, 25 mM EDTA pH 7.5, 250 mM NaCl, 0.5% SDS (sodium dodecylsulfate)	Extraction buffer used to extract the genomic DNA of infected basil tissue.

2.3 Primers used in this objective are listed in Table 5.2.

Table 5.2. Primers used for amplifying *P. belbahrii* ITS2 and basil β-tubulin using qPCR.

	Name	Sequence	Description
1	PbITS2-F	CTGAACAGGCGCTGATTG	qPCR amplifying *P. belbahrii* ITS2
2	PbITS2-R	GCAACAGCAAAGCCAATTC	
3	Obtubulin-F2	GCTTGCTGTCAATCTCATTCC	qPCR amplifying basil β-tubulin
4	Obtubulin-R2	TCTGGAACAGTGAGTGCTCTG	

3. METHODS

3.1 gDNA extraction for P. belbahrii sporangia

P. belbahrii sporangia were inoculated on 4-week-old basil genotype Dolly and harvested by swirling infected basil leaves in ddH_2O. The released sporangia were collected into 2 ml tubes

with screw caps, to which 400µl of glass beads (425-600µm) and 400µl of buffer AP1 (QIAGEN DNeasy Plant Mini Kit) were added. The sporangia were subjected to homogenization in high-speed bead beater (MP Biomedicals, FastPrep-24 Instrument). The 200µl of supernatant was then transferred into clean Eppendorf tubes after 1-min centrifugation at full speed. The pellet fractions were resuspended with another 200µl of AP1 buffer, and the supernatant was collected after centrifugation. A total 400µl of supernatant in each tube was used for DNA isolation following the manufacturer's instructions.

3.2 qPCR using P. belbahrii gDNA

Total 25ng *P. belbahrii* sporangia gDNA was diluted in a five-fold series to 8pg. The serially diluted gDNA were then used as templates for qPCR. The 20µl of PCR reaction was set up as follows: 1X SYBR Green mix, 250µM specific primers, and 5µl gDNA. qPCR program as described in Chapter 2 (3.4) was run on Bio-Rad C1000 Touch Thermal Cycler machine. The threshold cycle (Ct) numbers and corresponding log concentrations of gDNA were used to generate a regression line.

3.3 Pathogen inoculation and sample collection

4-week-old basil genotype Genovese was inoculated with sporangia of 4×10^4 spores/ml by dropping six 10µl drops on the second pair of true leaves. Samples were harvested at 0, 1, 2, 3, 4, 5 dpi. For each sample, 6 inoculated leaves from 3 plants were detached and wrapped in the aluminum foil and frozen in liquid nitrogen. 6 non-inoculated Genovese leaves were also collected. All the samples were stored at -80°C before use.

3.4 Crude gDNA extraction from infected basil leaves

Samples were ground in liquid nitrogen using precooled mortar and pestle. For better grinding, half of powder was discarded and the remaining was further ground into fine powder. A 400µl aliquot of crude DNA extraction buffer was added into a 1.5ml Eppendorf tube with around 100mg powder. The powder was resuspended in the buffer by vortexing. The 370µl of supernatant was collected following centrifugation at full speed for 2 min and subjected to a second round of centrifugation at full speed for 10 min. The resulting supernatant (350µl) was mixed with equal volume of 100% isopropanol and incubated at room temperature for 10 min.

Supernatant was discarded after 10-min centrifugation at full speed. The pellets were washed with 1ml of chilled 70% ethanol and centrifuged at full speed for 1 min. The supernatant was completely removed and the pellets were air dried at room temperature for around 15 min. The pellets were then resuspended in 100µl of AE buffer (QIAGEN DNeasy Plant Mini Kit) and stored at 4°C for overnight. The gDNA was quantified using Nanodrop 2000 spectrophotometer (Thermo Scientific) and diluted into 10ng/µl.

3.5 qPCR using crude gDNA

5µl (50ng) extracted gDNA for all the samples were individually mixed with 1X SYBR Green mix and 250µM specific primers (listed in **Table 5.2**) into the 20µl of PCR reaction. PCR program was conducted as described in 3.2 above. The pathogen biomass was quantified as the ratio of the amplification of *P. belbahrii* ITS2 to *O. basilicum* β-tubulin, which was calculated by $2^{-\Delta Ct}$ (the formula for ΔCt is the same as Chapter 2 (3.4, equation 1)).

4. RESULTS

The efficiency of the primers targeting the *P. belbahrii* ITS2 region was first determined by qPCR using the series-diluted gDNA from *P. belbahrii* sporangia as templates. This primer set achieved nearly linear amplification over the logarithm value of a 5 fold-diluted gDNA starting from 25ng (**Fig. 5.1),** indicating a very good correlation of the amplifications with the amount of templates. In practice, *P. belbahrii* needed to be quantified from infected plants. To assess whether the primers can be used to specifically quantify pathogen growth in infected tissues, gDNAs of non-infected Genovese basil tissues or inoculated tissues collected from a range of infection time courses up to 5 dpi were subjected to qPCR using the primer sets which amplified *P. belbahrii* ITS2 or *O. basilicum* β-tubulin, respectively. The relative pathogen biomass was calculated as the ratio of the amplification of *P. belbahrii* ITS2 to *O. basilicum* β-tubulin. No significant amplification was observed for gDNAs isolated from non-infected tissues using *P. belbahrii* ITS2 primers, suggesting that they are specific to the pathogen. On the infected Genovese basil plants, a consecutive accumulation of pathogen biomass over the time course up to 5 dpi was observed (**Fig. 5.2**), which seemed to correspond to the real disease development during infection. In total, the primer sets used in this objective were specific and efficient to quantify the growth of *P. belbahrii* on infected basil, and therefore was suitable to be utilized for

evaluating the altered disease effect resulted from *in planta* expression of effector candidate genes for dissecting their roles in disease development.

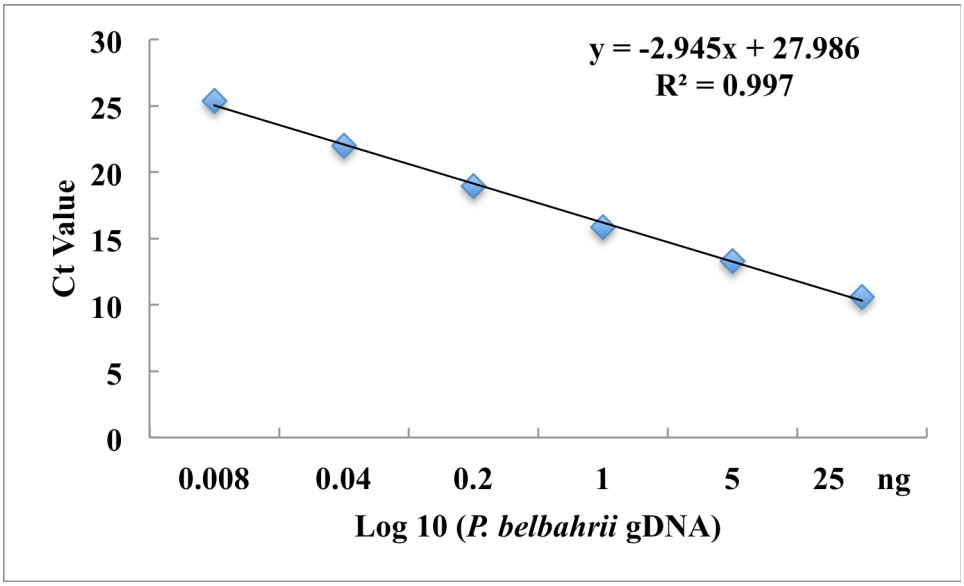


Figure 5.1. Correlation of *P. belbahrii* ITS2 qPCR Ct values with amounts of *P. belbahrii* gDNA. Ct values represent the average of three technical replicates, with error bars representing standard deviations. R^2: correlation coefficient.

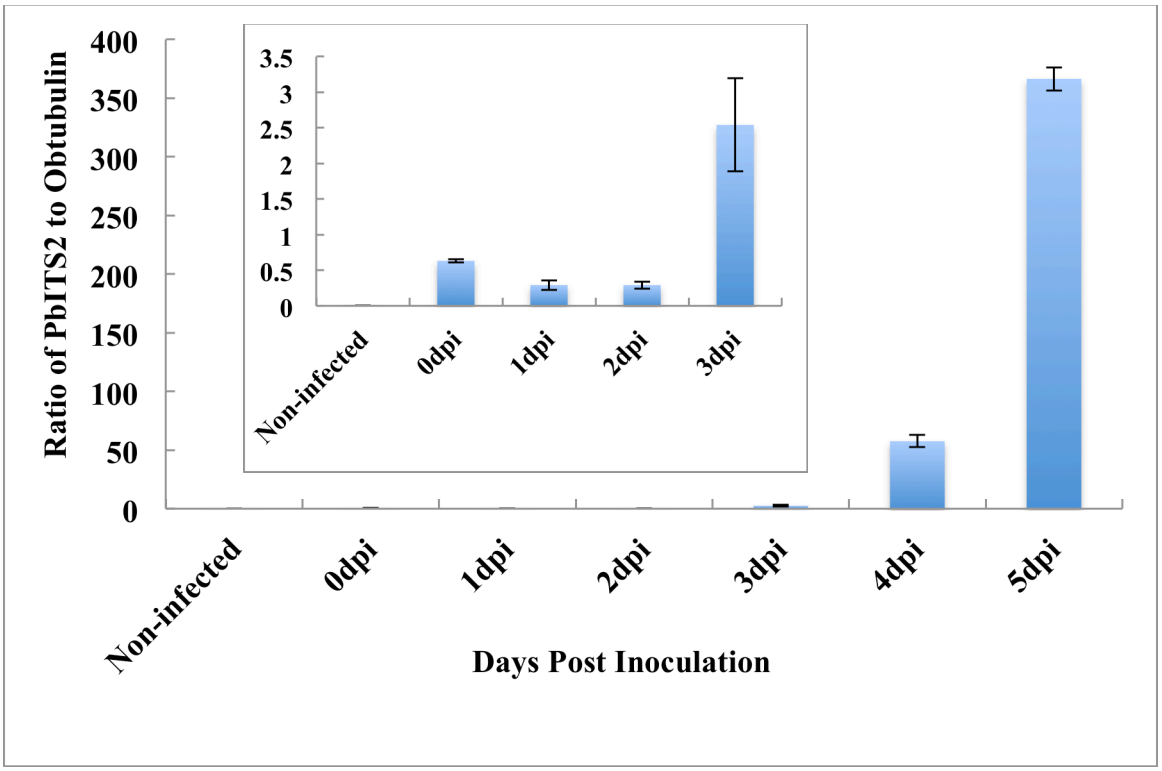


Figure 5.2. *P. belbahrii* growth on Genovese basil during infection time course. The growth was quantified as the ratio of the amplification of *P. belbahrii* ITS2 to *O. basilicum* β-tubulin ($2^{-\Delta Ct}$). The error bars represent ± standard deviations from three technical replicates.

CHAPTER 6

OBJECTIVE V: Determine the role of candidate effector gene PbEC2 in pathogenicity

1. INTRODUCTION

Similar to other obligate biotrophic pathogen, genetic identification of roles of *P. belbahrii* effector genes could be done through either overexpression of effectors in basil which often enhances the pathogen virulence (Asai et al., 2014) or host-induced gene silencing (HIGS) using effector gene-derived silencing construct which would reduce the pathogenicity (Nunes & Dean, 2012). In this objective, the overexpression and HIGS of PbEC2, the gene shown to be highly upregulated during infection and have the functional signal peptide, followed by determining its role in pathogenicity were conducted using both transient expression and stable transformation.

To perform transient overexpression through agro-infiltration, PbEC2 was cloned into pE1776-2HA under the transcriptional control of the superpromoter (Lee et al., 2007) and transformed into *A. tumefaciens* strain EHA105. For HIGS, DNA fragments of about 500bp were cloned into pHANNIBAL vector in both sense and antisense directions to generate inverted repeats spaced by a functional PDK intron which helps to form the hairpin loop (Wesley et al., 2001). To be able to express dsRNAs in basil, the hairpin RNA-forming fragment was cut from the recombinant pHANNIBAL vector and then cloned into pE1776 (Lee et al., 2007), the binary vector previously used to successfully express PbEC2 in basil. The gene-silencing construct was transformed into *A. tumefaciens* strain EHA 105 through electroporation. The resulting *Agrobacterium* strain was utilized to conduct transient HIGS and generate transgenic plants stably expressing HIGS construct. The integration of the transgene was confirmed and the expression level of the transgene in transgenic plants was also detected. The role of PbEC2 in pathogenicity is being determined by the pathogen infection assay.

2. MATERIALS

2.1 Basil and P. belbahrii strain

Basil genotype Genovese was used and grown under the same conditions as described in Chapter 4 (2.1). The *P. belbahrii* strain used and its maintenance were described in Chapter 2 (2.1). *P. belbahrii* inoculum preparation and inoculation of basil plants were performed as described in Chapter 2 (3.1 and 3.2).

2.2 Plasmids used in this chapter are listed in Table 6.1below.

Table 6.1. Plasmids used for PbEC2 overexpression and HIGS.

	Name	Reference	Description
1	pE1776	Lee et al., 2007	Plant binary vector for gene expression in plants
2	pE1776-2HA	Chapter 4	A modified plant binary vector for expressing proteins fused with 2HA tag at the C-termini in plants
3	pE1776-2HA-PbEC2	Chapter 4	Expression of PbEC2 fused with 2HA tag at the C-terminus in plants
4	pHANNIBAL	Jacobs et al., 1997	Vector used to generate inverted repeats for hairpin RNA formation
5	pHANNIBAL-EC2-RNAi(+)	This study	Recombinant pHANNIBAL vector cloned with sense PbEC2
5	pHANNIBAL-EC2-RNAi	This study	Recombinant pHANNIBAL cloned with sense and antisense PbEC2
6	pE1776-EC2-RNAi	This study	Expression of hairpin RNA of PbEC2 in plants

2.3 Primers used in this objective are listed in Table 6.2 below. Restriction enzyme sites are capitalized.

Table 6.2. Primers used for making constructs for overexpression and HIGS of PbEC2.

	Name	Sequence	Description
1	PbEC2-Fhind	5'-gcgAAGCTTatggtgggagacc ccatagcgaccacaa-3'	Clone PbEC2 into pE1776-2HA
2	PbEC2-Rspe	5'-gcgACTAGTggcgccagaggca tgaactt-3'	
3	Pbtubulin-F	5'-cggaaactggctgtgaactt-3'	qPCR for *P. belbahrii* β-tubulin
4	Pbtubulin-R	5'-caaagcacggtactgctgag-3'	
5	Obtubulin-F2	5'-gcttgctgtcaatctcattcc-3'	qPCR for basil β-tubulin using gDNA
6	Obtubulin-R2	5'-tctggaacagtgagtgctctg-3'	
7	EC2-RNAi-FXho	5'-gcgCTCGAGtgacggcccttaca ttaagaa-3'	Cloning of sense PbEC2 into pHANNIBAL
8	EC2-RNAi-RKpn	5'-gcgGGTACCggaacggtgtttag cgatgta-3'	
9	EC2-RNAi-FHind	5'-gcgAAGCTTtgacggcccttacat taagaa-3'	Cloning of antisense PbEC2 into pHANNIBAL
10	EC2-RNAi-RCal	5'-gcgATCGATggaacggtgtttag cgatgta-3'	
11	35Spromotor-F	5'-gtggattgatgtgacatctccac-3'	Colony PCR and sequencing for cloning of sense PbEC2 into pHANNIBAL
12	PDK-R	5'-cttcgtcttacacatcacttgtca-3'	
13	PDK-F	5'-gtcgaacatgaataaacaaggt-3'	Colony PCR and sequencing for cloning of antisense PbEC2 into pHANNIBAL
14	OCSterminator-R	5'-gatctgagctacacatgctcag-3'	
15	pMSP1-F1	5'-tggataaatagccttgcttcct-3'	Colony PCR and sequencing for pE1776-derived plasmids
16	pMSP1-R1	5'-gagcatggtctcagcgccggct-3'	

2.4 Bacteria

Competent cells of *Agrobacterium* strain EHA105 and *E. coli* strain DH5α used in this chapter were made and stored as described in Chapter 3 (2.4).

2.5 Media used in this chapter are listed in Table 6.3 below.

Table 6.3. Media used for culturing bacteria and stable transformation.

	Name	Components	Description
1	LB medium	2.5% LB broth miller (EMD Millipore) in ddH_2O (solid medium with additional 1.5% agar)	For growth of Agrobacteria and *E. coli* strain
2	SOB medium	2% w/v tryptone, 0.5% w/v Yeast extract, 8.56mM NaCl, 2.5mM KCl, 10mM $MgCl_2$ and 10mM $MgSO_4$	Medium for overnight culture of *E. coli* DH5α containing pHANNIBAL
3	Agrobacteria inoculation medium (IN)	MS (Sigma-Aldrich) + 3% sucrose +16.8µM (4mg/L) TDZ (thidiazuron), pH 5.7, supplemented with 200µM acetosyringone	For inoculation of Agrobacteria strain with basil explants
4	Callus and shoot induction medium (SI) for co-cultivation	MS + 3% sucrose + 16.8µM (4mg/L) TDZ + 0.8% agar, pH 5.7 supplemented with 200µM acetosyringone	For co-cultivation of Agrobacteria and basil explants
5	Callus and shoot induction medium (SI) for shooting	MS + 3% sucrose + 16.8µM (4mg/L) TDZ + 0.8% agar, pH 5.7 supplemented with Timentin (200 mg/L) and Kanamycin (30 mg/L)	For callus and shoot induction
6	Root induction medium (RI)	MS +3% sucrose + 0.8% agar + 0.01mg/L NAA (1-Naphthaleneacetic acid), pH 5.7 supplemented with Timentin (200 mg/L) and Kanamycin (30 mg/L)	For shoot elongation and root induction

3. METHODS

3.1 Vector for transient overexpression of PbEC2

The process was as described in Chapter 4 (3.2 and 3.3).

3.2 Vector construction for HIGS of PbEC2

3.2.1 Amplification of PbEC2 DNA fragments for cloning in sense and antisense directions

471 base pairs within PbEC2 sequence were chosen to design the specific primers with restriction enzyme sites added (XhoI and KpnI for sense direction; HindIII and ClaI for antisense direction). 1µl of extracted pE1776-2HA-PbEC2 plasmid were used as templates for PCR. Remaining reagents were also added into the 50µl of PCR reaction: 0.2mM dNTPs, 1X HF buffer, 0.4µM specific primers (EC2-RNAi-FXho and EC2-RNAi-RKpn for sense direction cloning; EC2-RNAi-FHind and EC2-RNAi-RCal for antisense direction cloning), and 1 unit of Phusion High-Fidelity DNA polymerase (NEB). PCR conditions started from an initial denaturation of 30 sec at 98°C, and then

followed by 35 cycles of thermocycling: 15 sec at 98°C for denaturation, 15 sec at 66°C for annealing, 30 sec at 72°C for elongation. The final step of elongation was at 72°C for 10 min. The resulted PCR products were purified using QIAGEN QIAquick PCR Purification Kit following the manufacturer's instructions.

3.2.2 Digestion of the PCR product for sense direction cloning and pHANNIBAL vector

E. coli strain DH5α containing pHANNIBAL plasmid was inoculated in SOB medium with 50mg/ml Cb shaking overnight and the plasmid extraction was performed using QIAGEN QIAprep Spin Miniprep Kit as described before. The digestion was conducted for the PCR product and pHANNIBAL plasmid. The reagents added into the 50µl of digestion reaction were: 30µl of purified PCR product or plasmid, 1X NEB Buffer 3.1, 10 units XhoI (NEB), 10 units KpnI (NEB), and ddH_2O up to 50µl. The reactions then were incubated at 37°C for 3 to 4 hrs.

3.2.3 Visualization of digestion product and gel extraction

Digestion products were resolved on 1% agarose gel by electrophoresis under 120 V and 500 mA for 35 min. The digested plasmid was excised from the gel and extracted using QIAquick Gel Extraction Kit.

3.2.4 Ligation of the sense PbEC2 DNA fragment to linear pHANNIBAL

The ligation was performed in 15µl of reaction with the ingredients added as follows: digested plasmid and sense PbEC2, 400 units of T4 DNA ligase (NEB), 1X ligase buffer. The reactions were incubated overnight at room temperature and stored at 4°C.

3.2.5 Transformation into E. coli strain DH5α through heat shock

The transformation was conducted as explained in Chapter 3 (3.1.5). 100µl of the resulting culture were spread on LB plates containing 50µg/ml Cb and grown overnight at 37°C. The single colonies appeared next morning were confirmed on new LB plates containing 50µg/ml Cb.

3.2.6 Colony PCR and sequencing

The colony PCR was conducted for the single colonies using 35S promotor-F and PDK-R listed in **Table 6.2**. The procedures for PCR and sequencing were performed as in Chapter 3 (3.1.6).

3.2.7 Isolation of recombinant pHANNIBAL inserted with PbEC2 fragment in sense direction

E. coli DH5α transformed with the recombinant plasmid pHANNIBAL-EC2-RNAi (+) was inoculated in SOB with Cb overnight and the plasmid was extracted next day.

3.2.8 Cloning of antisense PbEC2 fragment into pHANN-EC2-RNAi (+)

The step was as described for cloning sense PbEC2. The generated plasmid was named as pHANNIBAL-EC2-RNAi and the stock of *E. coli* DH5α containing this plasmid was stored at -80°C.

3.2.9 Digestion of pHANNIBAL-EC2-RNAi and pE1776

The plasmids of pHANNIBAL-EC2-RNAi and pE1776 were extracted using QIAGEN QIAprep Miniprep Kit. The digestion was conducted in the 50µl of digestion reaction containing the following ingredients: 30µl plasmid, 1X NEB Buffer 3.1, 10 units XhoI (NEB), 10 units HindIII (NEB), and ddH_2O up to 50µl. The reactions then were incubated at 37°C for 5 hrs.

3.2.10 Visualization of digestion product and gel extraction

The digested hairpin-forming DNA fragment and linearized pE1776 were visualized and extracted following the same descriptions in 3.2.3.

3.2.11 Ligation of hairpin-forming DNA fragment and linear pE1776

The ligation reaction and process were as mentioned in 3.2.4. The ligation product pE1776-EC2-RNAi was then transformed into *E. coli* DH5α competent cells as described in 3.2.5. The positive transformants were confirmed by colony PCR and sequencing using pMSP1-F1 and pMSP1-R1 as described before.

3.2.12 Transformation of pE1776-EC2-RNAi into Agrobacterium strain EHA105

pE1776-EC2-RNAi plasmid was extracted and transformed into EHA105 through electroporation as described in Chapter 4 (3.3.7). The transformants were also confirmed by colony PCR using pMSP1-F1 and pMSP1-R1 and the DMSO stock of a positive clone was stored at -80°C.

3.3 HIGS through stable transformation

3.3.1 Preparation of Agrobacterium and basil explants

EHA105 strain carrying pE1776-EC2-RNAi was cultured on LB plate containing 15μg/ml Rifampicin, 25μg/ml Gentamicin, and 50μg/ml Kanamycin at 28°C the day before use. On the day of transformation, the bacteria from the plate were suspended in IN medium with final OD_{600} of 0.6 and incubated at room temperature in dark with gentle shaking for 2 hrs. During the 2-hr incubation, the first true leaves of 3 to 4-week-old seedlings was surface-sterilized in 12% (v/v) Clorox for 5 min and rinsed 4 times in sterile water. Two small explants (with the diameter of around 5mm) were cut from the leaf base for each leaf using cork-borer.

3.3.2 Inoculation of explants with Agrobacteria

Excised explants were immersed in the *Agrobacterium* solution in 50ml falcon tube for 30 min in the dark. The explants were taken out and excess solution was removed with sterile filter paper.

3.3.3 Co-cultivation on callus and shoot induction medium

Explants (around 15/petri dish) were transferred with abaxial side facing the medium to SI medium supplemented with 200μM acetosyringone in cell culture dishes (100 cm * 20 cm) (Greiner bio-one, Austria). The dishes were kept in the dark at 25°C for 3 days.

3.3.4 Shoot regeneration and selection of transgenic shoots

Explants were transferred to SI medium supplemented with Timentin (200 mg/L) and Kanamycin (30 mg/L) in the dishes and grown for 2 weeks in the dark at same

temperature. A secondary or further subculture into fresh SI medium may or may not have been performed depending on the shoot development condition.

3.5.5 Shoot elongation and root regeneration

The regenerated shoots were cut and transferred to RI medium supplemented with Timentin (200 mg/L) and Kanamycin (30 mg/L) in 190ml containers (Greiner bio-one, Austria) and grown for 2 weeks in the dark at 25°C and returned to condition with a 12-hr photoperiod afterward. The shoots with developed roots were transferred to fresh RI medium with Timentin and Kanamycin until ready for acclimatization.

3.5.6 Acclimatization of the plantlets to green house condition:

Plantlets with well-developed root systems were removed from culture, rinsed in water to remove media, and transferred into moistened soil (SunGro Horticulture Sunshine Mix #4) in 5" AZ green plastic pots (Niu Nursery, Honolulu, HI). The pots were maintained 100% humidity for 3 to 4 d in a tray covered with dome and placed in the growth chamber at 25°C with a 12-hr photoperiod. Humidity was gradually reduced over a 3 to 4-d period and the plants were then transferred to the greenhouse.

3.3.7 Confirmation of integration of transgene through PCR

About 50mg leaf tissue from generated basil lines was frozen in liquid nitrogen and ground using blue pestle (Axygen Scientific Inc). Crude gDNA was then extracted from the powder as described in Chapter 5 (3.4). 1µl gDNA was used as PCR template. Regular PCR using primers for amplifying the inserted PbEC2 DNA fragment in antisense direction were conducted with the 25µl of reaction contained the following ingredients: 200µM dNTPs, 1X Taq buffer, 1.5mM $MgCl_2$, 0.5 µM specific primers (EC2-RNAi-FHind and EC2-RNAi-RCal listed in **Table 6.2**) and 1 unit of Taq DNA polymerase (Lamda biotech). PCR conditions started from an initial denaturation of 2 min at 94°C, followed by 35 cycles of thermocycling: 15 sec at 94°C for denaturation, 30 sec at 54°C for annealing, and 1 min at 72°C for elongation. The final step of elongation took place at 72°C for 5 min. PCR products were visualized on a 1% agarose gel by electrophoresis under 120 V and 500 mA for 35 min.

3.3.8 Expression level of RNAi construct in transgenic basils

6 leaf disks (with the diameter of around 8 mm) from 2 leaves were collected using corker-borer for each of 13 transgenic lines and non-transformed wide type. Total RNA were extracted from the samples and processed into cDNA synthesis as previously described. The cDNAs (approximately 25ng) were used as the template for qPCR using primers that amplify the PDK intron (PDK-F and PDK-R), the sequence within the PbEC2 silencing-targeting region (PbEC2-F and PbEC2-R), and basil β-tubulin (Obtubulin-F2 and Obtubulin-R2) for normalization. PCR was conducted as described in Chapter 5 (3.2).

4. RESULTS

Transient overexpression and HIGS of PbEC2 were conducted by infiltrating *Agrobacteria* EHA105 harboring pE1776-2HA-PbEC2 or pE1776-EC2-RNAi, respectively, into the leaves as described previously. After two days, the infiltrated areas were then inoculated with *P. belbahrii* sporangial suspension. At 5 days post-inoculation, the treated leaves were harvested and gDNAs from the samples were extracted and used as templates for qPCR. The pathogen biomass was quantified as the ratio of the amplification of *P. belbahrii* β-tubulin (by using primers Pbtubulin-F and Pbtubulin-R listed in **Table 6.2**) to *O. basilicum* β-tubulin (by using primers Obtubulin-F2 and Obtubulin-R2 listed in **Table 6.2**). The effect resulted from neither the transient overexpression nor HIGS of PbEC2 was consistent when comparing the pathogen biomass from treatments with the one from control (data not shown). Several possible reasons for this inconsistency were listed as follows: 1) the primers used for pathogen quantification (Pbtubulin-F and Pbtubulin-R) are not as sensitive as the ones used in Chapter 5 (PbITS2-F and PbITS2-R); 2) the time point (5 dpi) for colleting samples may be too late to detect the effect of PbEC2 as duration of PbEC2 expression could be short; 3) the leaf disks from treated leaves collected as samples may not represent the effect on whole leaves. Future experiments are needed to optimize the factors mentioned above.

To conduct HIGS through stable transformation, the silencing construct of PbEC2 was used to transform Genovese, a susceptible genotype shown to be amenable for transient expression.

Total 13 transgenic lines (labeled as #0 to #13, #6 was absent) were generated through *Agrobacterium*-mediated transformation (**Fig. 6.1**). The integration of the transgene was confirmed by PCR amplifying the antisense PbEC2 fragment using crude gDNA from transgenic plantlets (**Fig. 6.2**). All the lines displayed the band with estimated size of around 500bp, indicating the insertion of the transgene (**Fig. 6.2**). The expression level of the transgene was detected by qPCR amplifying the PDK intron as well as a partial region of PbEC2 within the hairpin RNA-forming construct using cDNA synthesized from total RNA of transgenic lines. Varied expression levels of the inserted transgene were observed (**Fig. 6.3**) and the ones with relatively high level of expression were subjected to propagation by seed planting. Pathogen infection assays will be performed on the second generation of the transgenic lines to determine the feasibility of HIGS in *P. belbahrii*-basil pathosystem and the role of PbEC2 in pathogenicity.

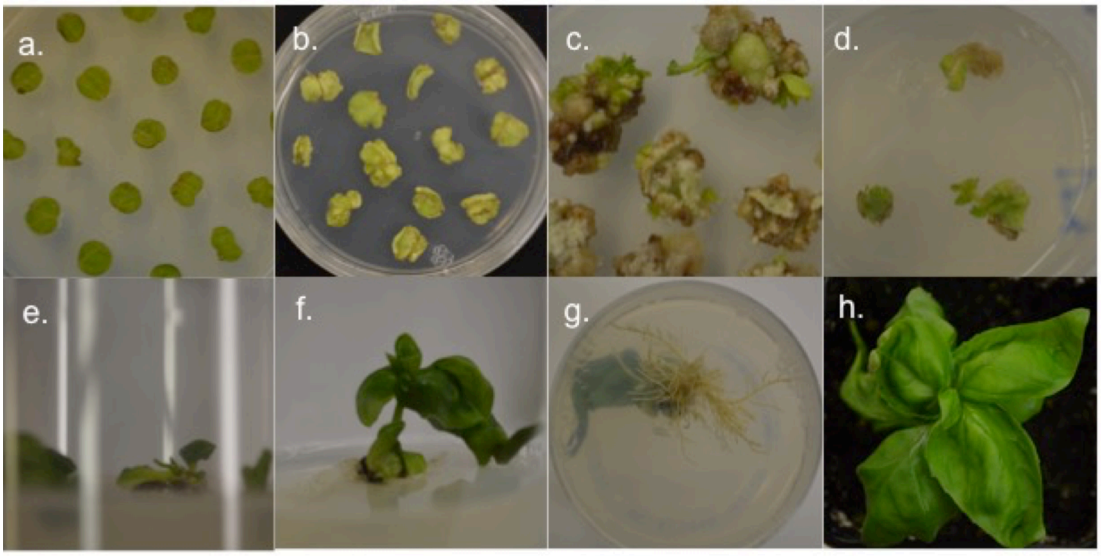


Figure 6.1. Stages in generation of transgenic basil. **a.** Co-cultivation of explants and *Agrobacteria* on callus and shoot induction medium (SI) under dark for 3 days after inoculation of explants with *Agrobacteria*. **b.** Callus formation on SI after 14 days under dark. **c.** Shoot regeneration on SI after 14 days or longer under dark. **d.** Regenerated shoots on root induction medium (RI) under dark for 14 days. **e.** Shoots on RI under light for 14 days or longer. **f and g.** Shoot elongation (f) and root development (g) on RI under light. **h.** Acclimatization of plantlet in soil.

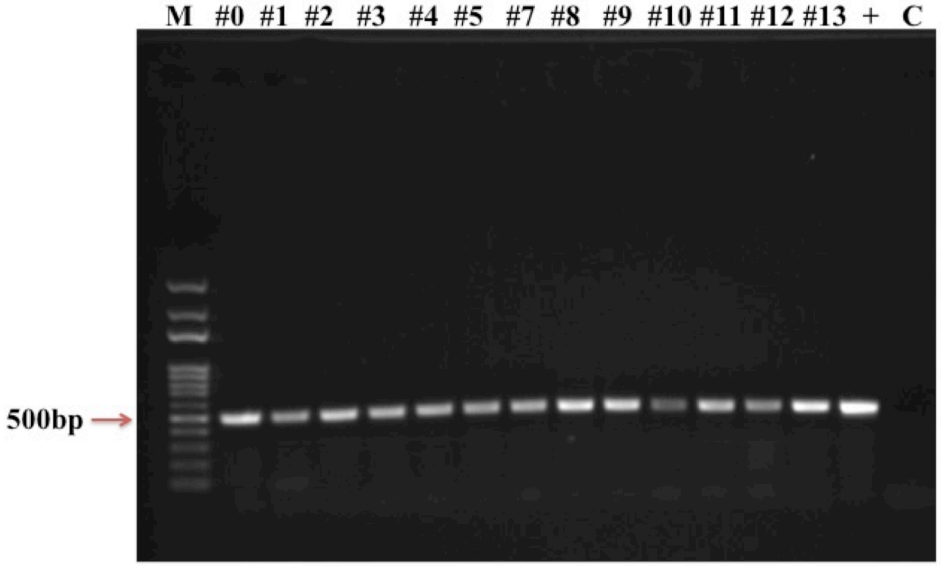
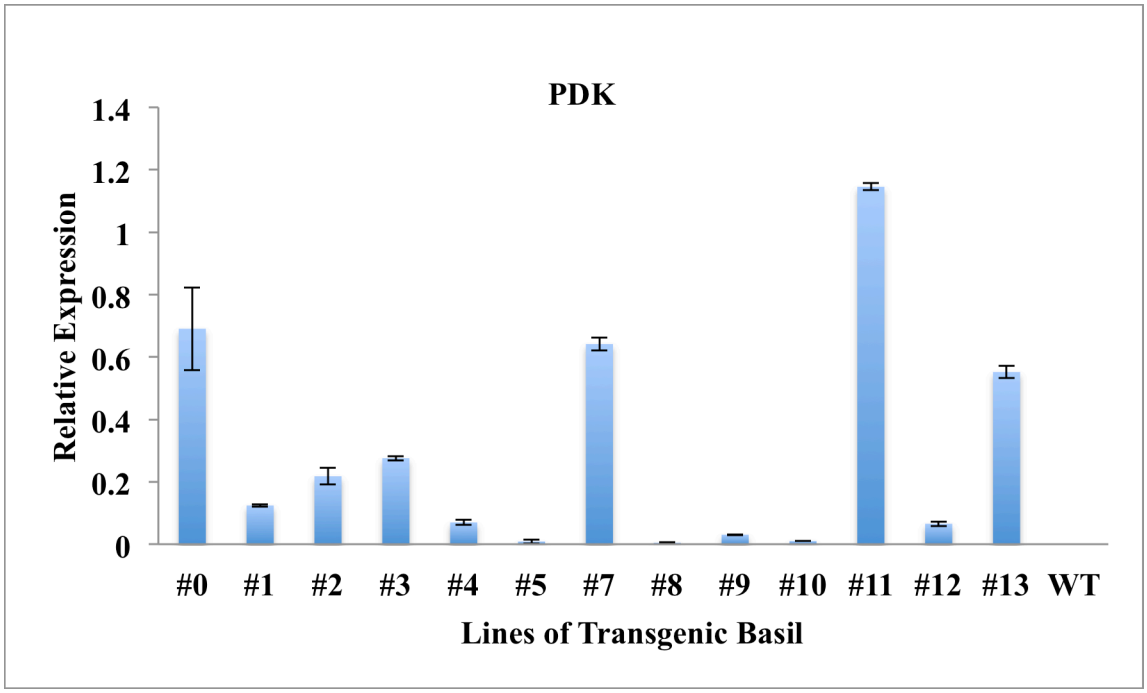


Figure 6.2. Detection of integration of PbEC2 HIGS construct in transgenic basil lines. Agarose gel of PCR products of the antisense PbEC2 fragment amplified from basil lines (from #0 to #13). +: PCR product with the pure pE1776-EC2-RNAi plasmid as template. C, no template control.

A.



B.

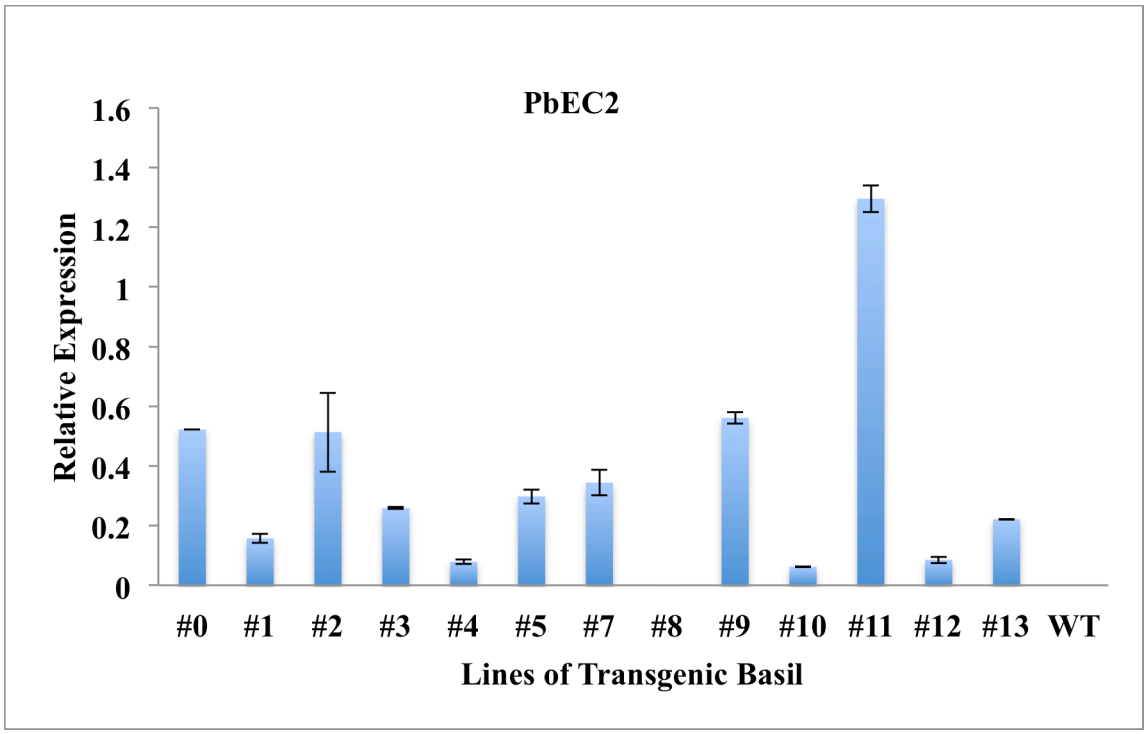


Figure 6.3. Expression level of the PbEC2 hairpin RNA in transgenic lines. **A**. The expression level of PDK intron in different lines. **B**. The expression level of PbEC2 in different lines. The relative expression level is normalized by constitutively expressed *O. basilicum* β-tubulin. The error bars represent ± standard deviation from three technical replicates. WT: wild type.

CHAPTER 7

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