Investigations of Barley Powdery Mildew Effectors (CSEPs) in the Non-host Plant Wheat and Host Plant Barley

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This thesis is a presentation of the author's own work and all other work is appropriately referenced

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Abstract

Blumeria graminis f. sp. *hordei* is a biotrophic obligate fungus that can survive and cause powdery mildew disease exclusively on living barley plants. Over 500 Candidate Secreted Effector Proteins (CSEPs), which are considered to be secreted by the fungal feeding structure haustoria, have been identified. Some of the CSEPs, such as CSEP0064 and CSEP0264, were found to contribute to the pathogen virulence.

Wheat is resistant to *Blumeria graminis* f. sp. *hordei*-This is a form of non-host resistance. Recently, a *Pseudomonas fluorescens* based effector-to host analyser strain (EtHAn) was developed as a modified T3SS, which can deliver foreign effectors into plants. In this study, I used the EtHAn strain to deliver CSEP0064 and CSEP0264 into wheat, and screened for effector-triggered responses in a mapping population of wheat (WAGTAIL). Although the bacterium itself induced pattern-triggered immunity (PTI) in wheat, three cultivars (Rialto, Abbot and Madrigal) were capable of consistently recognizing and differentiating introduced CSEP0064 or CSEP0264, leading to an enhancement of EtHAn-triggered PTI.

Subsequently, I tested whether the recognition of EtHAn-delivered effectors in these three lines can affect the susceptibility of wheat to subsequent fungal infection. The results showed that the recognition of introduced CSEPs triggered systemic resistance in Rialto and Madrigal, whereas induced systemic susceptibility in Abbot, to the adapted pathogen *Blumeria graminis* f. sp. *tritici.* Delivered CSEPs did not affect systemic wheat immunity to sebsequent infection of the non-adapted pathogen *Blumeria graminis* f. sp. *hordei.* These results suggest that either wheat host resistance and non-host resistance are controlled by different systemic signalling pathways or basal plant immunity is sufficient to restrict non-host pathogen invasion and additional up-regulation of systemic immunity does not provide any additional advantage.

In parallel, the expression of CSEP0064 in host plant barley was also investigated. In this study, I observed that CSEP0064 is detectable by western blotting in heavily infected barley leaves and isolated haustoria. Unexpectedly, a protein with an apparent molecular mass around 200 kDa was recognized by the anti-CSEP0064 antibody on western blots. Here, I refer to this protein as 'Big CSEP'. This 'Big CSEP' was detected in fungal structure-containing materials only. TFMS-mediated deglycosylation successfully removed this 'Big CSEP' signal, suggesting that the 'Big CSEP' may be a hyper/*O*-glycosylated CSEP0064, or a glycosylated complex containing CSEP0064.

Large-scale shotgun proteomics have been performed on *Blumeria graminis* f. sp. *hordei*, leading to the identification of some haustoria-exclusive effectors including CSEP0064. Here, MRM-MS was used to detect and quantify CSEP0064 in different fractions of infected barley leaf. An enzymatic method was conducted to isolate haustoria from infected barley epidermis. GAPDH (*Blumeria graminis* f. sp. *hordei*) and GAPDH (*H. vulgare*) were used as reference for fungal and plant proteins. CSEP0064 was more abundant than GAPDH (*Blumeria graminis* f. sp. *hordei*) in isolated haustoria and the plant cytoplasm fractions. Moreover, CSEP0064 was four times more abundant than GAPDH (*H. vulgare*) in the plant cytoplasm fraction. Finally, the results demonstrate the secreation of CSEP0064 from haustoria and the uptake of CSEP0064 by the host cell.

Abbreviations

Abbreviation	Description
Avr	avirulence
A/B	acrylamide/bisacrylamide
ABC	ammonium bicarbonate
ACN	acetonitrile
B-A	borane-ammonia
Bgh	*B. graminis* f. sp. *hordei*
Bgt	*B. graminis* f. sp. *tritici*
BECs	*Blumeria* effector candidates
β-ME	β-mercaptoethanol
BTH	benzothiadiazole
BSA	bovine serum albumin
cDNA	complementary DNA
Co-IP	Co-Immunoprecipitation
CSPs	cold-shock proteins
CSEPs	Candidate Secreted Effector Proteins
CHAPs	propanesulfonate
cv.	cultivar
DAB	diaminobenzidine
DA	dehydroabietinal
DNase	deoxyribonuclease
DTT	dithiothreitol
dpi	days post inoculation
DSS	disuccinimidyl suberate
EF-Tu	elongation factor Tu
ED	epidermis
EDTA	ethylenediaminetetraacetic acid

Endo H	endoglycosidase H
ER	endoplasmic reticulum
EP	epiphytic
EPI	enhanced product ion
ETI	effector-triggered immunity
EtHAn	effector-to-host analyser
EV	empty vector
eEF1G	elongation factor 1 gamma protein
f. sp.	*forma specialis*
GST	glutathione-S-transferase
GTPase	enzymes bind to guanosine triphosphate
G3P	glycerol-3-phophate
HA	haustoria
HR	hypersensitive response
IP	immunoprecipitation
IPTG	isopropyl-β-D-1-thiogalactopyranoside
HRP	horseradish peroxidase
HIGS	host induced gene silencing
ISR	induced systemic resistance
ISS	induced systemic susceptibility
INA	2,6-dichloroisonicotinic acid
JA	jasmonic acid
LRR	leucine-rich repeat
LC	liquid chromatography
MRM	multiple reaction monitoring
MALDI-TOF	matrix-assisted laser desorption/ionization time-of-flight
MAMP	microbe-associated molecular pattern
MAGIC	multiparent advanced generation intercross

MeSA	methyl salicylate
MS	mass spectrometry
NB	nucleotide-binding
NHR	non-host resistance
NPR	nonexpressor of pathogenesis-related genes
PTM	post-translational modification
PRR	pattern recognition receptor
PR	pathogenesis related
PAMP	pathogen-associated molecular pattern
PTI	PAMP-triggered immunity
PVPP	polyvinylpolypyrrolidone
Pst	*Pseudomonas syringae* pv. *tomato*
PNGase F	Peptide-N-Glycosidase F
PVDF	polyvinylidene fluoride
PVX	Potato Virus X
PBS	phosphate buffered saline
Pip	pipecolic acid
PCD	programmed cell death
qPCR	quantitative real-time PCR
QconCAT	quantification concatemer
QQQ	triple quadrupole
RPM	revolutions per minute
RNase	ribonuclease
RT	room temperature
ROS	reactive oxygen species
SA	salicylic acid
SAR	systemic acquired resistance
SAS	systemic acquired susceptibility
SUMO	small ubiquitin-like modifier

SDS	sodium dodecyl sulfate
PAGE	polyacrylamide gel electrophoresis
Slp	secreted LysM protein
SIS	systemic induced susceptibility
SRM	selected reaction monitoring
TCA	trichloroacetic acid
TFMS	trifluoromethanesulfonic acid
TCEP	tris (2-carboxyethyl) phosphine
TFA	trifluoroacetic acid
Tris	trisaminomethane
TGase	transglutaminase
T3SS	type three secretion system
TMV	tobacco mosaic virus
WAGTAIL	Wheat Association Genetics for Trait Advancement and Improvement of Lineages
Y2H	yeast-2-hybrid

1 Introduction

1.1 Food security and sustainability

The primary challenge for worldwide agriculture is providing nutritious and adequate food for the growing number of global human population(Curtis and Halford, 2014). An increase of 70% in food production will be needed by 2050 (Food and Agricultural Organization of the United Nations (FAO), 2009). Moreover, the growth of agricultural production needs to be sustainable, without compromising the environmental quality and public health (Shiferaw et al., 2011, Krattinger and Keller, 2016).

1.2 Wheat and barley

Wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) are two of the most economically important cereal crops worldwide (FAO). Wheat is the staple food for more than half of the world's population, while barley is one of the most dominant crops in Europe (Gent et al., 2014, Haberer and Mayer, 2015). Their production can be utilized for animal feed as well as human nutrition (Reynolds et al., 2016).

1.3 Crop improvement

In natural environments, crop plants are threatened by various diseases such as powdery mildews, resulting in significant yield losses (Kwak and Weller, 2013). Currently, the most environmentally friendly approach for disease control is breeding resistant cultivars (Vleeshouwers and Oliver, 2014, Komaromi et al., 2016). Apart from utilizing host plant resistance genes, the researchers and breeders also realized the potential of using non-host resistance genes to produce more durable and broad-spectrum resistant cultivars (Lee et al., 2016, Tang et al., 2017). It is therefore crucial to understand both host and non-host aspects of important pathogens.

1.4 Biotrophic fungal pathogens

When a phytopathogenic fungus establishes a long-term nutrient exchange relationship with living cells, rather than killing the cells of its host, it is called a biotroph and the interaction is biotrophic (Spanu and Panstruga, 2017, Spanu and Kamper, 2010). The successful colonization of biotrophic fungi is often accompanied by the development of specialized feeding structures such as haustoria and intracellular invasive hyphae (Yi and Valent, 2013, Dodds et al., 2009, Giraldo et al., 2013). Both haustoria and intracellular hyphae are surrounded by the plant-derived membranes (Figure 1.1), extrahaustorial membrane and extrainvasive hyphal membrane, respectively (Chaudhari et al., 2014, Lo Presti et al., 2015). Although extrahaustorial membrane and extrainvasive hyphal membrane are continuous with the plasma membrane, they appear to lack some common plant plasma membrane proteins (Lo Presti et al., 2015, Khang et al., 2010, Xing et al., 2013).

Biotrophic fungi cause many worldwide destructive diseases on cereals, such as rusts, powdery mildews and maize smut.

1.5 Effectors

The last decade has seen dramatic progress in discovery/identification of effectors (Bialas et al., 2017). Effectors are generally defined as pathogen secreted factors that manipulate host immune responses in favor of the pathogen, function either inside cells (cytoplasmic effectors and nuclear effectors) or in the apoplast (apoplastic effectors) (Figure 1.1) (Varden et al., 2017, Stergiopoulos and de Wit, 2009, Rovenich et al., 2014). Apoplastic effectors often possess a cysteine-rich C-terminus and a signal peptide. These effectors are usually small and function primarily as protease inhibitor proteins (Varden et al., 2017). For instance, the Avr2 effector of fungal pathogen *Cladosporium fulvum* is thought to suppress host defense through inhibiting specific host proteases (Stergiopoulos and de Wit, 2009). Similar to apoplastic effectors,

cytoplasmic effectors have a secretion signal at the N terminus, and multi-domain (e.g., uptake or translocation domain) toward the C terminus. Additionally, some conserved amino acid motifs have been identified in cytoplasmic effectors, such as the RxLR (arginine, any amino acid, leucine and arginine) motif in oomycete effectors (Sonah et al., 2016). Compared to apoplastic effectors, cytoplasmic effectors target host signalling components at a more complex level. They translocate to host cytoplasm and function in other subcellular compartments. Nuclear effectors usually localize in the nucleus to affect the host immune system. Some also show nuclear-cytoplasmic localization, such as *Meloidogyne incognita* effector MilSE6. Nuclear effectors have the potential to reprogram host transcription or shut off the master switches of the host immune machinery to contribute to pathogen fitness and effectiveness (Chaudhari et al., 2014). The mechanisms by which effectors traffic to plant cells are still unclear. A variety of mechanisms have been suggested due to the high diversity in the sequence and structure of effectors (Figure 1.1). Although the term effector mostly means effector protein, the definition of it is evolving with our understanding of plant-microbe interactions (Guyon et al., 2014). In a broader sense, all proteins, metabolites and other molecules that play roles during infection can be referred to as effectors (Sonah et al., 2016, Lo Presti and Kahmann, 2017, Liu et al., 2009).

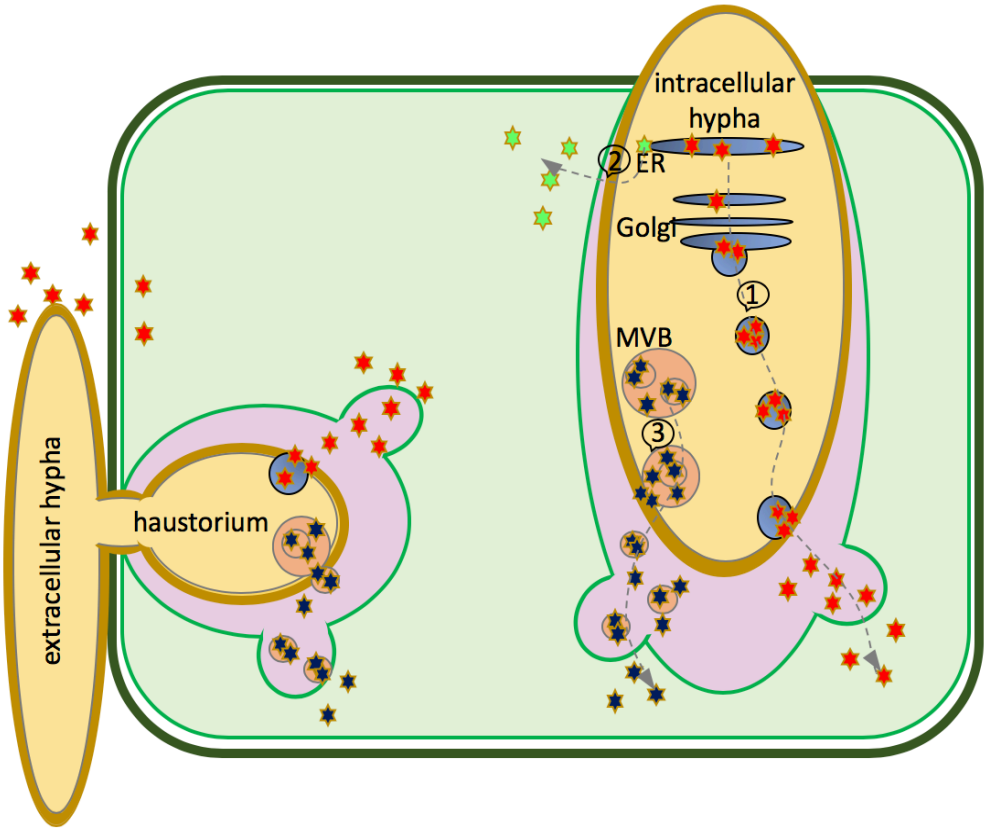

Figure 1.1 Scheme of effector secretion and translocation from filamentous pathogens into plant cells. Plant cell wall: dark green line. Pathogen cell wall: brown line. Pathogen plasma membrane: grey line. The extracellular hyphae colonize outside plant cells while the haustoria and intracellular hyphae are surrounded by the plant-derived membrane (green line). Both extracellular hyphae and invasive intracellular feeding structures can secret effectors (stars, red/black/light green). Effectors can be secreted through different routes: (1) ER-Golgi secretory route, (2) the Golgi-independent route and (3) an unconventional multivesicular bodies (MVB)-dependent route. Effectors (red stars) secreted following route (1) can translocate into the plant cytosol (light green background) via endocytosis, while those (black and light green stars) secreted following routes (2) & (3) can enter plant cells via endocytosis or in association with fungal exosomes. How extracellular hyphae-secreted effectors enter the plant cytosol is unknown. Adapted from Lo Presti and Kahmann (2017).

1.6 Plant immune system (blurred PTI-ETI dichotomy)

It is generally accepted that the plant innate immune system can be broadly divided into two layers (Qi et al., 2011, Gill et al., 2015). The plant basal immunity involves and depends on the recognition of pathogen-associated molecular patterns (PAMPs, also known as microbe-associated molecular patterns, MAMPs) by pattern recognition receptors (PRRs) located in plasma membrane (Gouveia et al., 2016, Segonzac and Zipfel, 2011). PAMP-triggered immunity (PTI) is the first layer of plant immune responses and is associated with cell wall fortification in the form of callose deposition, oxidative burst and up-regulation of defense-related genes (Liu et al., 2016a, Zipfel and Oldroyd, 2017, Felix and Boller, 2003). However, PTI is not sufficient to counter all pathogens. Some pathogens can deploy an arsenal of effectors to manipulate the host physiology to promote infection (Crabill et al., 2010). In turn, plants use other receptors (referred to as R proteins) to recognize and interact with pathogen-secreted effectors directly or indirectly (Delaunois et al., 2014, Hurt et al., 2014). Effectors that are recognized by corresponding R proteins are called avirulence (Avr) proteins (Lim and Kunkel, 2004). Moreover, most, but not all, R proteins comprise a nucleotide-binding domain (NB) and a leucine-rich repeat (LRR) domain (Lee and Yeom, 2015, Cesari, 2017). The recognition of an effector by a cognate plant R protein leads to effector-triggered immunity (ETI), which is the second layer of plant immunity (Boyd et al., 2013, Johansson, 2015). Typically, ETI is associated with hypersensitive response (HR), which is a form of programmed cell death (PCD) at the infection site (Heath, 1998, Toruño et al., 2016). The traditional PTI-ETI dichotomy described above has been widely used (Jones and Dangl, 2006).

However, a growing body of evidence indicates that the discrimination between PTI and ETI cannot be maintained strictly. In the classic PTI-ETI dichotomy, PAMPs predicted are commonly structural molecules, for instance, fungal chitin (Miya et al., 2007) and bacterial flagellin (Gómez-Gómez and Boller, 2002). Unlike effectors that are expressed to facilitate

infection on specific plant species (Selin et al., 2016, Park and Seo, 2015), PAMPs were considered to be broadly conserved and act as general elicitors (Huckelhoven, 2007, Thomma et al., 2011). Nevertheless, several studies have shown that some PAMPs or effectors possess characteristics that are similar to each other (Stracke et al., 2002). For example, Pep-13, which constitutes a surface-exposed fragment of a cell wall transglutaminase (TGase) and activates defense in parsley and potato, is only conserved among *Phytophthora* species (Brunner et al., 2002), demonstrating its narrow distribution. Additionally, a few PAMPs display defense-eliciting activity in a narrow range of plants (Lapin and Van den Ackerveken, 2013). Another example is that the bacterial cold-shock proteins (CSPs) and elongation factor Tu (EF-Tu) are recognized exclusively by Solanaceae (Wang et al., 2016) and Brassicaceae (Kunze et al., 2004), respectively. On the other hand, some R proteins carry typical features of PRRs (Thomma et al., 2011). For example, tomato R protein Ve1 mediates resistance to two distinct fungal species, suggesting that the elicitor of Ve1 is broadly conserved as a PAMP, which in turn implies that Ve1 is a PRR (Fradin et al., 2009). Apart from their distribution, the functions of PAMPs and effectors were thought to be diverse. PAMPs were considered to facilitate the survival and fitness of pathogens, whereas effectors contribute to virulence (Bigeard et al., 2015, Alfano and Collmer, 2004). Nevertheless, several examples indicate that some PAMPs also play important roles in virulence. For instance, specific *P. syringae* pv *tabaci* flagellin mutants affected in elicitor activity have been shown to display reduced virulence in plants due to reduced motility (Ishiga et al., 2005). Furthermore, some PAMPs including harpins and ethylene-inducing xylanase can induce PCD (Xie and Chen, 2000, Ron and Avni, 2004), which was considered to be a hallmark of ETI (Thomma et al., 2011). Conclusively, it appears to be a continuum rather than a distinct separation between PTI and ETI (Denance et al., 2013, Vetter et al., 2016).

1.7 *Blumeria graminis*

Powdery mildews (Ascomycetes of the order Erysiphales) are among the most common and destructive diseases of many dicot and monocot plants (Troch et al., 2014, Krattinger and Keller, 2016). They can infect and colonise on leaves, stems and fruits of their hosts (Lipka et al., 2008). *Blumeria graminis* is a group of biotrophic fungi which can cause powdery mildews on cereals and grasses (Huckelhoven and Panstruga, 2011). These fungi display strict host specialization, among which eight *formae species* (f.sp.) have been found to infect particular host plants (Menardo et al., 2016, Walker et al., 2011). For example, *B. graminis* f. sp. *hordei* infects and establishes a compatible interaction exclusively with barley, whereas *B. graminis* f. sp. *tritici* can only survive on wheat (Glawe, 2008, Dean et al., 2012). *B. graminis* has become a model organism to study phytopathogenic obligate biotrophs (Menardo et al., 2017, Orton and Brown, 2016).

1.7.1 Asexual lifecycle

The asexual lifecycle of *B. graminis* commences with a conidiospore landing on the host plant surface (Figure 1.2). The two most crucial steps in the course of its lifecycle are the successful invasion by the penetration peg and the accommodation of terminal infection structure haustorium (Glawe, 2008, Huckelhoven, 2007, Both et al., 2005). Like other biotrophic fungi which have haustoria, *B. graminis* haustoria are surrounded by extrahaustorial membrane, and therefore remain separated from the host cell cytoplasm (Thordal-Christensen et al., 2018, Catanzariti et al., 2006, Wang et al., 2009). Although the molecular mechanisms by which haustoria develop need to be further elucidated, it is clear that haustoria are not only functional in nutrient uptake and signalling exchange, but also affect host immunity by secreting numerous effectors (Spanu, 2012, Spanu and Panstruga, 2017, Franceschetti et al., 2017).

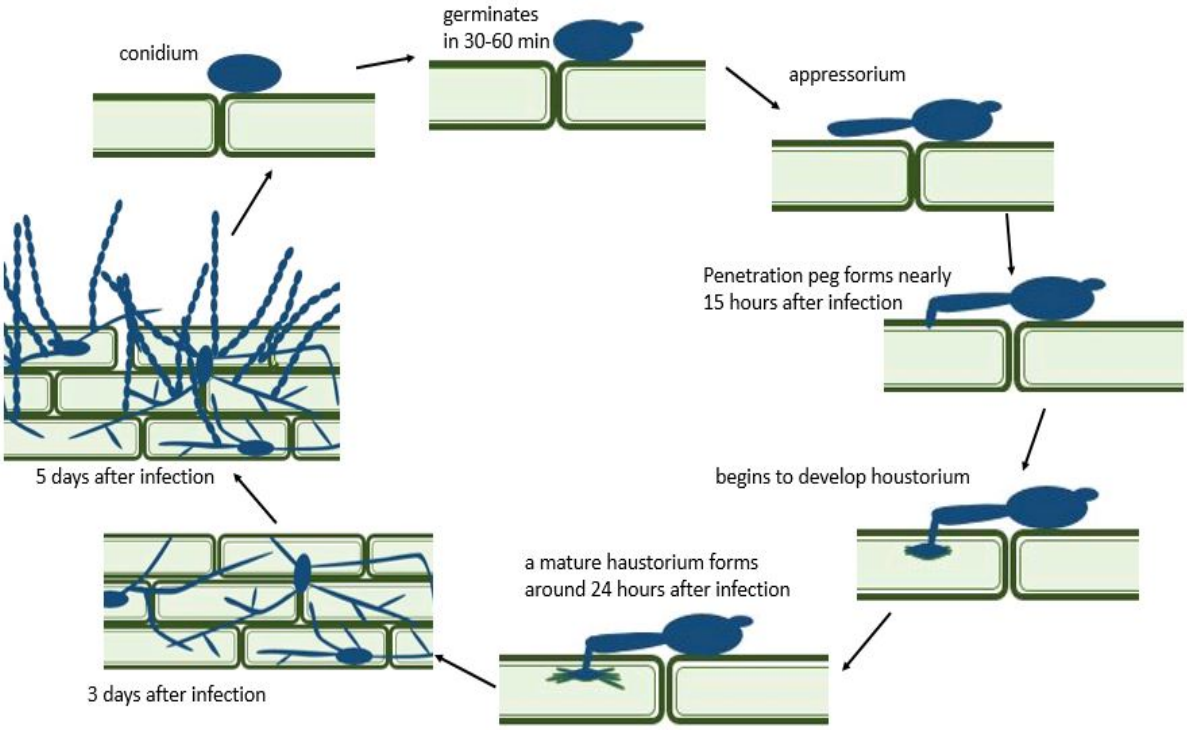

Figure 1.2 Asexual lifecycle of *Blumeria graminis*. Once landing on a susceptible host plant, the conidiospore begins to germinate within one hour. Following spore germination, the appressorium develops from the secondary germ tube. Then the appressorium initiates a penetration peg in the following several hours to penetrate into the plant epidermal cell. Once inside, the penetration peg enlarges and forms the feeding structure haustorium. The haustorium becomes mature and fully functional nearly 24 hours post inoculation. As the infection progresses, new colonies develop on the leaf surface and produce conidia, which can be seen by naked eyes from about 3 days after infection. Adapted from Both *et al.* (2005).

1.7.2 Sexual reproduction

The sexual reproduction in *B. graminis* is initiated by production of gametangia and ends with the release of ascospores (Braun et al., 2006). Sexual reproduction is thought to contribute to the occurrence of new races that may overcome resistance (Wicker et al., 2013, Komínková et al., 2016).

1.8 Barley powdery mildew

Barley powdery mildew, caused by *B. graminis* f. sp. *hordei*, may result in yield losses as large as 40% on barley (McGrann et al., 2014).

1.8.1 B. graminis *f. sp.* hordei *genome*

It is estimated that the genome size of *B. graminis* f. sp. *hordei* isolate DH14 is ~120 Mb, which is nearly four times larger than the median of other closely related ascomycetes (Spanu et al., 2010, Spanu, 2014). The huge size of *B. graminis* f. sp. *hordei* genome is due to the proliferation of retrotransposons that generate massive repetitive DNA (Spanu et al., 2010, Kusch et al., 2014). Despite its large size, *B. graminis* f. sp. *hordei* genome harbors a relatively small number of protein coding genes (~ 7000), which is lower than most of other ascomycete phytopathogens (Schmidt and Panstruga, 2011, Bourras et al., 2018, Frantzeskakis et al., 2018). The reduction in its gene content can be partially explained by the dramatic decrease in genes encoding some specific pathways, such the secondary metabolite biosynthesis, which is consistent with its biotrophic nature (Spanu, 2012, Bindschedler et al., 2016, Bourras et al., 2016). Additionally, the loss of many genes that are conserved in other ascomycetes also contribute to the *B. graminis* f. sp. *hordei* gene reduction. For example, the genes encoding the biosynthesis of thiamine and enzymes for nitrate assimilation are absent in *B. graminis* f. sp. *hordei* (Spanu et al., 2010, Kusch et al., 2014). Besides isolate DH14, the genomes of two additional *B. graminis* f. sp. *hordei* isolates A6 and K1 have also been sequenced (Hacquard et al., 2013).

1.8.2 CSEPs

1.8.2.1 Identification of CSEPs

A critical outcome of whole genome sequencing is the identification of putative effector proteins. More than 500 Candidates for Secreted Effector Proteins (CSEPs) that constitute ~ 8%

of the total annotated genes have been identified in *B. graminis* f. sp. *Hordei.* These putative effectors have no homologies outside powdery mildews, have a signal peptide for secretion and lack recognizable transmembrane domains (Spanu et al., 2010, Pedersen et al., 2012, Hacquard et al., 2013). In addition to genome information, the discovery of barley powdery mildew effectors also benefit from the transcriptome and proteome studies (Both et al., 2005, Bindschedler et al., 2009, Godfrey et al., 2010). For example, a group of *Blumeria* effector candidates (BECs) that defined as proteins specifically associated with and secreted from haustoria were discovered through proteome studies of infected barley epidermal materials (Bindschedler et al., 2009, Pliego et al., 2013, Bindschedler et al., 2011). Moreover, there is almost complete overlap between CSEPs and BECs, with five exceptions including broadly conserved virulence proteins BEC1005 and BEC1019 (Bindschedler et al., 2016, Whigham et al., 2015).

1.8.2.2 CSEP0064 and CSEP0264

Most of the predicted CSEPs were found predominantly up-regulated in haustoria (Pedersen et al., 2012). CSEP0064 and its paralog CSEP0264 are two of them (they share 75% nucleotide identity). In the same study, 72 CSEPs were predicted to have a ribonuclease-like fold, among which CSEP0064 and CSEP0264 are similar to RNAse T1 (*Aspergillus oryzae*) and RNAse U2 (*Ustilago sphaerogena*), respectively. The structure of CSEP0064 (Figure 1.3) has recently been confirmed experimentally (Spanu et al., 2018). By comparing the proteomes of the haustoria-containing epidermis and sporulating hyphae, Bindschedler *et al.* (2011) identified 71 haustoria-exclusive BECs including BEC1054 and BEC1011, which are identical to CSEP0064 and CSEP0264, respectively (Bindschedler et al., 2011). In addition, host induced gene silencing (HIGS) of 50 BECs led to highly variable effects on fungal virulence by affecting the haustoria development. Eight of them were found to result in significant reduction of haustoria formation, including BEC1054 and BEC1011 (Pliego et al., 2013). Thus, they are

suggested to act as *bona fide* effectors that contribute to *B. graminis* f. sp. *hordei* virulence. A more recent study measured the transcript abundance of four CSEPs, including CSEP0064 and CSEP0264, suggesting that they may play roles in multiple stages of the fungal infection process (Pennington et al., 2015).

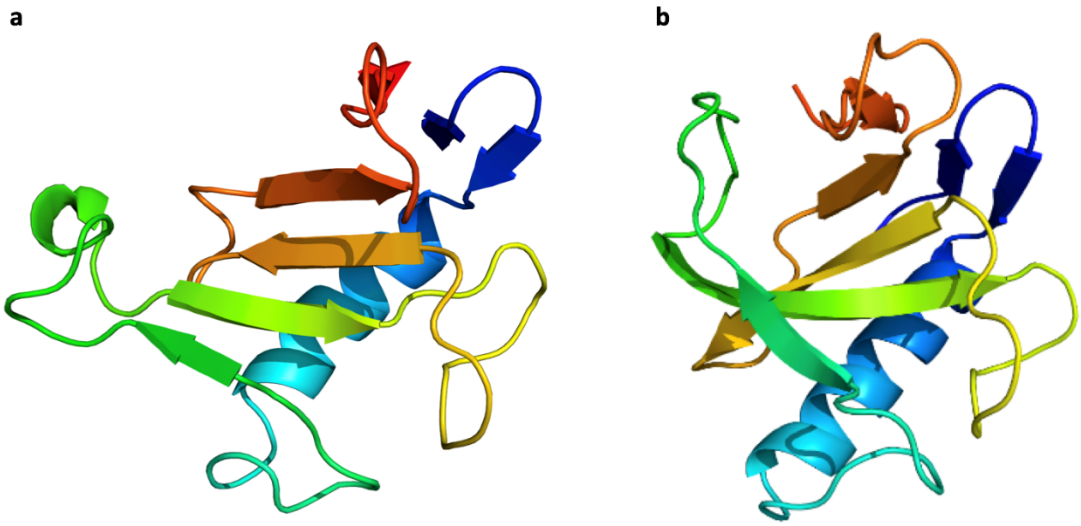

Figure 1.3 CSEP0064 and RNase T1 possess remarkably similar structures. The sequences of CSEP0064 and RNase T1 in PDB files were uploaded onto PyMol2 to visualise the structures. The colour spectrum displays the N-terminus (blue) to C-terminus (red). (a) CSEP0064 from *B. graminis* f. sp. *hordei*. (b) RNase T1 from *A. oryzae*.

CSEP0064 and CSEP0264 were chosen to be investigated in this thesis because they are two effectors that play central roles in *B. graminis* f. sp. *hordei* virulence on barley and are relatively well-studied (Pliego et al., 2013, Pennington et al., 2015, Pennington et al., 2016).

1.8.2.3 Host targets of CSEPs

Several recent studies identified the putative host targets of some CSEPs using the yeast-two-hybrid method. CSEP0055 contributes to the aggressiveness of the fungus, and interacts with barley pathogenesis-related protein families, PR1 and PR17 (Zhang et al., 2012). Two sequence-unrelated CSEPs, CSEP0105 and CSEP0162, seem to promote fungal virulence by

interacting with barley stress-related small heat shock proteins Hsp16.9 and Hsp17.5 (Ahmed et al., 2015, Ahmed et al., 2016). Moreover, CSEP0064 (syn. BEC1054) targets a barley pathogenesis-related protein (PR5) isoform, an elongation factor 1 gamma protein (eEF1G), a malate dehydrogenase (MDH) and a glutathione-S-transferase (GST) (Pennington et al., 2016). But, it is unclear how the interactions between CSEP0064 and its host targets are affecting the barley immune or metabolism systems.

As mentioned above, it is important to investigate the mode of action of effectors in both host plants and non-host plants. Although many large-scale genomic, transcriptomic and proteomic studies have been carried out to predict the expression and localization of CSEPs (Pliego et al., 2013, Pedersen et al., 2012, Bindschedler et al., 2009, Bindschedler et al., 2016), studies of the expression of CSEPs in host barley plants at a protein level is still scarce.

2 Aims and objectives of this thesis

Aim 1: To investigate whether CSEP0064 and CSEP0264 can be recognized in non-host plant wheat and the role they play in regulating wheat immune system upon recognition.

Specific objective (1): Deliver two CSEPs into non-host wheat plant using a modified T3SS EtHAn, and screen for effector-specific responses.

Specific objective (2): Test if the recognition of CSEPs can induce the susceptibility change (locally/systemically) in wheat to subsequent infection with non-adapted pathogen *B. graminis* f. sp. *hordei*

Specific objective (3): Test if the recognition of CSEPs can trigger the susceptibility change (locally/systemically) in wheat to subsequent infection with adapted pathogen *B. graminis* f. sp. *tritici*

Aim 2: To investigate the expression and localization of CSEP0064 in host plant barley.

Specific objective (1): Utilize western blot to detect CSEP0064 in infected barley leaves and dissected tissues.

Specific objective (2): Identify the possible modification of CSEP0064 using western blot.

Specific objective (3): Utilize MRM-MS to quantitatively analyse the expression of CSEP0064 in different dissected tissues of infected barley leaves.

3 Screening for CSEP-specific responses in the WAGTAIL mapping population

3.1 Introduction

Plants are continuously threatened by numerous potentially pathogenic microorganisms. As a result, they have evolved a range of defense mechanisms they deploy to protect themselves.

3.1.1 Non-host resistance

The distinction between PTI and ETI notwithstanding, the outcome of successful plant immunity is resistance to pathogens. If a plant species is resistant to all isolates of a given microorganism which is pathogenic to other species, this phenomenon is called non-host resistance (NHR) (Schulze-Lefert and Panstruga, 2011, Dido et al., 2016). NHR is triggered by PRR-mediated recognition in distantly-related plants, whereas in evolutionary closely-related plants the recognition of non-host effectors plays a vital role in NHR (Schulze-Lefert and Panstruga, 2011).It means similar or even the same factors, which can initiate plant defense system, may be involved in NHR and host resistance (Zellerhoff et al., 2010, Adlung et al., 2016). To give an example, *P. syringae* pv. *tomato* effectors AvrA and AvrD are recognized by *R* genes of nonhost plant soybean *Rpg2* and *Rpg 4,* respectively (Ashfield et al., 1995, Dou et al., 2008). Despite overlaps between NHR and host resistance, NHR is considered to be a more complex, durable and effective form of plant resistance, which is nevertheless still poorly understood (Mysore and Ryu, 2004, Heath, 2000). Several components that contribute to NHR have been described in particular plant-pathogen species. For instance, the plant cytoskeleton provides an important physical barrier of barley, wheat, *Arabidopsis* and tobacco against various invading pathogens including barley and wheat powdery mildews (Yun et al., 2003). Again in *Arabidopsis*, phytoalexin (Thomma et al., 1999) and saponins (Wees et al., 2003) play

important roles against the non-adapted pathogen *Alternaria brassicicola,* but it is unclear whether they play similar roles against other non-host pathogens. Additionally, plant hormores have also been demonstrated to mediate NHR (Deshmukh et al., 2006). Mutations in methyl salicylate esterase gene *SABP2*, which catalyzes the conversion of methyl salicylic acid into salicylic acid, compromised NHR of *Nicotiana* to *P. syringae* pv. *phaseolicola* (Chigurupati et al., 2016). Salicylic acid contributes to NHR in pea plant against *Fusarium solani* by inducing pathogenesis-related (*PR)* gene activation and plant DNA damage (Hadwiger and Tanaka, 2017). However, it is not known to which extent these components play a role in NHR.

More recently, Mysore and Rue (2004) suggested to classify NHR into two types according to the absence/presence of resistance phenotypes: type I and type II. Type I NHR shows no visible symptoms such as necrosis, whereas type II is always associated with a visual marker, necrosis or cell death (Mysore and Ryu, 2004, Oh et al., 2006). The two types of NHR triggered are dependent on both the pathogen species and plant species: a given pathogen species can trigger both type I and type II NHR on different plant species. For example, *P. syringae* pv. *phaseolicola* elicites type I NHR in *Arabidopsis* (Lu et al., 2001), while type II NHR in tobacco (Lindgren et al., 1986). One single plant species exhibit both types of NHR against different pathogens. For example, *N. benthamiana* shows type I NHR against *X. campestris* pv. *campestris* but exhibits type II against *P. syringae* pv. *tomato* (Peart et al., 2002). Moreover, type I NHR typically employs the defense factors to respond to general elicitors such as PAMPs, whereas type II is associated with HR which is similar to that triggered by ETI (Senthil-Kumar and Mysore, 2013, Niks, 2014). Although the genes involved in two types of NHR have not yet been clearly characterized, a study of expression profiles of 12 *PR* genes during type I and type II resistance of *N. tabacum* indicated that the same set of defense-related genes may involve in two types of NHR (Oh et al., 2006).

With regards to NHR against *B. graminis,* most of the investigations were carried out on the model plant *Arabidopsis*. NHR to *B. graminis* has been divided into two phases, pre-invasion (also called pre-haustoria in some studies) includes early stages (spore germination, appressorium formation and penetration) of fungal development before haustoria and post-invasion (post-haustoria) starts with haustoria establishment (Lipka et al., 2005, Ellis, 2006). During the interactions between *Arabidopsis* and *B. graminis* f. sp. *hordei*, more than ninety percent of landed spores fail to penetrate into the plant cell and are arrested by infection-induced papilla. The others which can evade pre-invasive NHR begin to form haustoria inside plant cell but encased in callose. The cells processing post-haustorial NHR finally undergo HR (Lipka et al., 2008). It was then suggested that *B. graminis* f. sp. *hordei* fails to penetrate and colonize in *Arabidopsis* due to the absence of some host-specific transport components or effector repertoire (Lipka et al., 2008, Huckelhoven and Panstruga, 2011). Additionally, Collins *et al.* (2003) screened for mutants that increased the rate of successful penetration of *B. graminis* f. sp. *hordei* in *Arabidopsis* and identified three penetration genes *PENs*. Similarly, two genes *Required for mlo-specific resistance Ror1* and *Ror2* are involved in resistance of barley against non-adapted soybean rust pathogen, where *Ror2* is an ortholog of *PEN1* (Huckelhoven et al., 2000). More intriguingly, *Ror2* also play roles in the interactions between barley and adapted *B. graminis* f. sp. *hordei* in pre-haustorial phase (Assaad et al., 2004). These significant overlaps indicate that there is a potential mechanistic link between host resistance and NHR (An et al., 2017, Collins et al., 2003). Similarities between NHR and host resistance were also observed in barley against adapted pathogen *B. graminis* f. sp. *hordei* and non-adapted pathogen *B. graminis* f. sp. *tritici*. For example, H_2O_2 accumulates in the interactions both between barley- *B. graminis* f. sp. *hordei* and barley- *B. graminis* f. sp. *tritici* (Huckelhoven et al., 1999, Huckelhoven et al., 2001). It is also worth noting that the

identification of many effectors and *R* genes is mainly based on transient expression assay (Giesbers et al., 2017, Adlung et al., 2016, Lee et al., 2014).

3.1.2 Effector-to-host analyser strain (EtHAn)

Various transient expression assays have been developed and widely used as a powerful tool for expressing and analyzing isolated or paired fungal effectors in host and non-host plants (Bashandy et al., 2015). Over the last few decades, the most intensively used system is the *Agrobacterium*-mediated assay, which relies on transient expression of T-DNA vectors in plants (Du et al., 2014, Bhaskar et al., 2009). However, this assay has not successfully been employed in monocot plants, such as wheat and barley. A single-cell transient assay that employs biolistic delivery of plasmid DNA into epidermal cells has been reported in barley and rice (Bai et al., 2012), but this assay is considered to be labour-intensive and incapable of high-throughput expression and screening. Some fungal and oomycete phytopathogens such as *Ustilago maydis* and *Magnaporthe oryzae* are now genetically transformable, thus enabling more effector-focused studies in their host plants such as maize and rice (Petre et al., 2014). Nevertheless, there has been no evidence showing that they can deliver foreign effectors into non-host plants. An alternative is utilizing bacterial type three secretion system (T3SS) to deliver fungal effectors into plants; this has been described in several studies, for instance, delivering oomycete effectors into *Arabidopsis* and *Nicotiana* by *P. syringae* pv. *tomato* DC3000 (Sohn et al., 2007, Fabro et al., 2011).

Thomas *et al*. (2009) developed a *Pseudomonas fluorescens* based effector-to-host analyser strain (EtHAn) to deliver directly foreign effectors into plants. The EtHAn is constructed by integrating the competent *hrp/hrc* cluster, which encodes the bacterial T3SS, from *P. syringae* pv. *syringae* into *P. fluorescens. P. fluorescens* is a soil bacterium that is not pathogenic to plants. EtHAn system can transport non-host bacteiral and fungal effectors into *Arabidopsis* and *Nicotiana* (Thomas et al., 2009, Thiebeauld et al., 2017). Moreover, it has been confirmed

that this assay is functional in wheat leaves. For example, Yin and Hulbert (2011) first reported the possibility of introducing effectors into wheat. They successfully observed hydrogen peroxide in wheat, which is induced by two avirulence proteins delivered by EtHAn (Yin and Hulbert, 2011). The success of using EtHAn in wheat was then further verified by delivering two avirulence proteins (AvrBs2 and AvrRpm1) into wheat and observing the resultant HR (Upadhyaya et al., 2014). Importantly, it was found that EtHAn-delivered wheat tripe rust effector PEC6 suppresses bacteria-triggered PTI in wheat leaves (Liu et al., 2016a). All this work suggested a promising way of studying barley powdery mildew (non-transformable) effectors (CSEPs) in non-host plant wheat.

3.1.3 The WAGTAIL wheat mapping population

A growing number of multiparent mapping populations have been developed over the past few years in different plant species. Examples include the *Arabidopsis* multiparent RIL population (Huang et al., 2011) and multiparent advanced generation intercross (MAGIC) population of wheat (Gardner et al., 2016). Unlike the studies focusing on single diseases, a mapping population program is designed for investigating the resistance genes of multiple diseases simultaneously. In particular, a Wheat Association Genetics for Trait Advancement and Improvement of Lineages (WAGTAIL) mapping population is developed to create an integrative map of four wheat diseases, including powdery mildew. The WAGTAIL consists of 480 wheat lines provides more random allelic combinations, which makes it particularly suitable for gene-to-gene interaction analysis.

To uncover the roles that effectors play in NHR more efficiently, there are an increasing number of studies which investigate effectors in multiple non-host plant accessions simultaneously to screen for the effector-mediated responses. For instance, two non-host downy mildew effectors are introduced into different lettuce accessions and elicit HR in half of them (Giesbers et al., 2017). Another study performed between downy mildew effectors and

lettuce included 34 effectors and 129 lines (Stassen et al., 2013). Similarly, fifty-four *Phytophthora infestans* effectors are transiently expressed in different pepper accessions, then two of the effectors induce cell death in the majority of total accessions (Lee et al., 2014). In the context of *B. graminis* f. sp *hordei*, the activities of CSEPs have not been demonstrated in non-host plants, such as wheat. In the present study, EtHAn system is used to deliver CSEPs into non-host plant wheat and screen for the effector-triggered responses. The WAGTAIL population is chosen to contribute phenotypic diversity in screening.

3.2 Objectives

1. To deliver two relatively well-studied *B. graminis* f. sp. *hordei* effectors CSEP0064 and CSEP0264 into non-host wheat plants using EtHAn system.

2. To screen for effector-specific responses by visual inspection of the symptoms.

In future, this would lead to the identification of the gene/genes that specify the recognition of effectors in wheat.

3.3 Materials and Methods

3.3.1 Plant materials

Wheat seedlings (*Triticum aestivum*) were grown in a 16-hour light/ 8-hour dark cycle in a temperature-control (20 ℃) room. There were 441 wheat lines from WAGTAIL numbered 1-441 and one extra variety Cerco included in this project.

3.3.2 Bacterial materials and growth conditions

The EtHAn strain (derived from *P.fluorescens*) was grown at 30 ℃ in Lysogeny-Broth (LB) with chloramphenicol (30 μg/ml). EtHAn-EV (EtHAn strain carrying empty vector) was cultured with chloramphenicol (30 μg/ml) and gentamycin (25 μg/ml). EtHAn-AvrPmaA1 (EtHAn carrying the avirulence protein AvrPmaA1 was grown in LB with chloramphenicol

(30 µg/ml) and kanamycin (25 µg/ml). All bacteria were stored at -80 ℃ in LB (with 20% glycerol) till needed.

3.3.3 Construction of EtHAn plasmids

Two candidate effector (CSEP0064 and CSEP0264) coding sequences were transferred into the destination vector (pDEV6) by Gateway LR cloning (ThermoFisher Scientific, US) from respective entry clones (pENTR221). After the LR reaction, the expression clone carried the T3SS and effector genes as shown in a schematic diagram below.

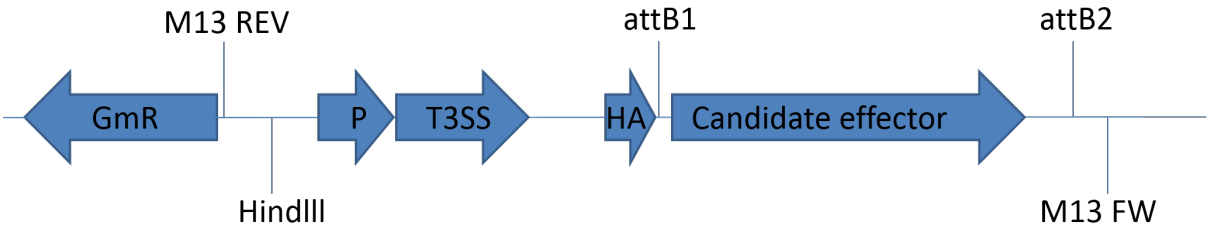

Figure 3.1 Schematic diagram of the candidate effector expression vector. GmR, gentamicin resistance gene; M13 REV/FW, M13 primer binding sites; P, T3 promoter; T3SS, T3SS secretion signals; HA, single HA epitope tag; attB1/B2, recombination site of GATEWAY cloning.

3.3.4 EtHAn conjugation

3.3.4.1 Preparation of heat-shock competent P. fluorescens cells

The *P. fluorescens* EtHAn strain was grown in 5 ml of LB medium with appropriate antibiotics overnight at 30 ℃, shaking (250 rpm). On the second day, 300 µl of the overnight culture was added to 20 ml LB and incubated at 30 ℃ with antibiotics until the culture reached an OD_{600} = 1.8-2.0. The culture was chilled for at least 20 min on ice and then centrifuged at 3000 *g* for 10 min in a 50 ml Falcon tube. The bacterial pellet was then suspended in 10 ml of pre-chilled 0.1 M $CaCl_2$ and kept on ice for 30 min. The cell suspension was centrifuged for another 10min, and then resuspended in 1 ml of pre-cooled 0.1 M $CaCl_2$. While incubating cell

suspension on ice, 33 µl of DMSO was added and mixed by gentle swirling. After 15 min's incubation, another 33 µl DMSO was added. Finally, the suspension was distributed in 100 µl aliquots into chilled freezing tube (1.5 ml). After snap-freezing in liquid nitrogen, the tubes were stored at -80 ℃.

3.3.4.2 Heat-shock transformation

The competent cells were thawed on ice for at least 20 min before mixed with 50 ng of plasmid DNA by pipetting. The mixture was then incubated on ice for 30 min. Heat shock was carried out at 42 ℃ for exactly 2 min followed by quenching on ice immediately for another 2 min. Then the mixture was mixed with 250 µl of pre-warmed SOB medium and incubated at 33 ℃ for 3-4 hours. Once recovery completed, 100 µl of the mixture was plated onto an appropriate selective plate. All transformants were examined by colony-PCR and plasmid sequencing (GATC Biotech, Germany).

3.3.5 EtHAn-infiltration

Fresh overnight culture of bacteria was prepared in 10 ml LB media every time before infiltration. The bacteria suspension was centrifuged at 3000 *g* for 10 min at room temperature before resuspending the pellet with 10 ml 10mM $MgCl_2$. Then the bacterial suspension was centrifuged again at 3000 *g* to remove the supernatant. Finally, the pellet was suspended in 10mM $MgCl_2$ (infiltration solution) to a final OD_{600} = 1.0. The inoculum can be left at room temperature for up to four hours before use.

The first leaves of 10-day wheat seedlings or both first and second leaves of 10-day barley seedlings were infiltrated with EtHAn carrying target effectors using 1 ml needleless syringe. All infiltrations were done at 3 cm from the top of leaf. The boundaries where the infiltration liquid extended to were labelled with a marker pen. EtHAn-AvrPmaA1 was included as a positive control to show the HR on wheat after infiltration. EtHAn-EV was included as the

negative control. For statistical and quantification analysis, three leaves of each wheat lines were infiltrated every time for each effector/control. Total three independent replicates (rounds of infiltration) were included. Plants after infiltration were maintained at the same conditions described above.

3.3.6 Visualization of H_2O_2

Accumulation of H_2O_2 in wheat and barley leaves was detected using 3, 3' – diaminobenzidine (DAB). Leaves were cut 4 days post infiltration and immersed in 1 mg/ml DAB solution (pH was adjusted to 3.8 with HCl, a low pH is necessary for thoroughly dissolving DAB in H_2O) in a 50 ml Falcon tube. The leaves were incubated in DAB staining buffer at room temperature for 8 hours with gentle shaking to ensure enough DAB was taken up. After incubation, leaves were boiled for 15-30 min in 96% ethanol to bleach out the chlorophyll. Then 96% ethanol was replaced by 70% ethanol and allow for incubating overnight to completely bleach the background, while retaining the dark brown precipitate formed by DAB when detecting H_2O_2. The accumulation of H_2O_2 in leaves can be directly visible to the naked eyes after overnight bleaching. Leaves at this stage can be stored at room temperature for up to 3-4 days.

3.3.7 Quantification of response intensity

MATLAB was used for the image processing. As shown in the images below, the true-colour image (Figure 3.2.a) was converted to a grey-scale image (Figure 3.2.b) by eliminating the hue and saturation information. Only the luminance information was retained after converting and all the luminance had their corresponding values from 0 to 255. Therefore, infiltrated and responding area were more luminant will have a higher value than the non-infiltrated or non-responding area (see Figure 3.2.b&c). The response intensity of infiltrated area of each single leaf was calculated by subtracting the 'background intensity', which is the intensity of non-infiltrated area on the same leaf. The standard deviation was calculated for three leaves of each

treatment. Responses on effector-infiltrated leaves were compared with EV-infiltrated leaves. Student's *t*-test was used to ascertain if there was a significant difference between these two treatments (Figure 3.2.c&d). Only effector-infiltrated leaves which showed a $p \leq 0.05$ were considered to have a significantly higher level of chlorosis than EV-infiltrated, and were considered to be the 'positive' responding lines. To exclude the interference caused by syringe damage, the infiltrated area was divided into spreading region (area above and below the wound caused by injection) and localised region (wound and inside). Response intensities of spreading and localized region were compared between EV-infiltrated leaves and effector-infiltrated leaves separately (Figure 3.2.d).

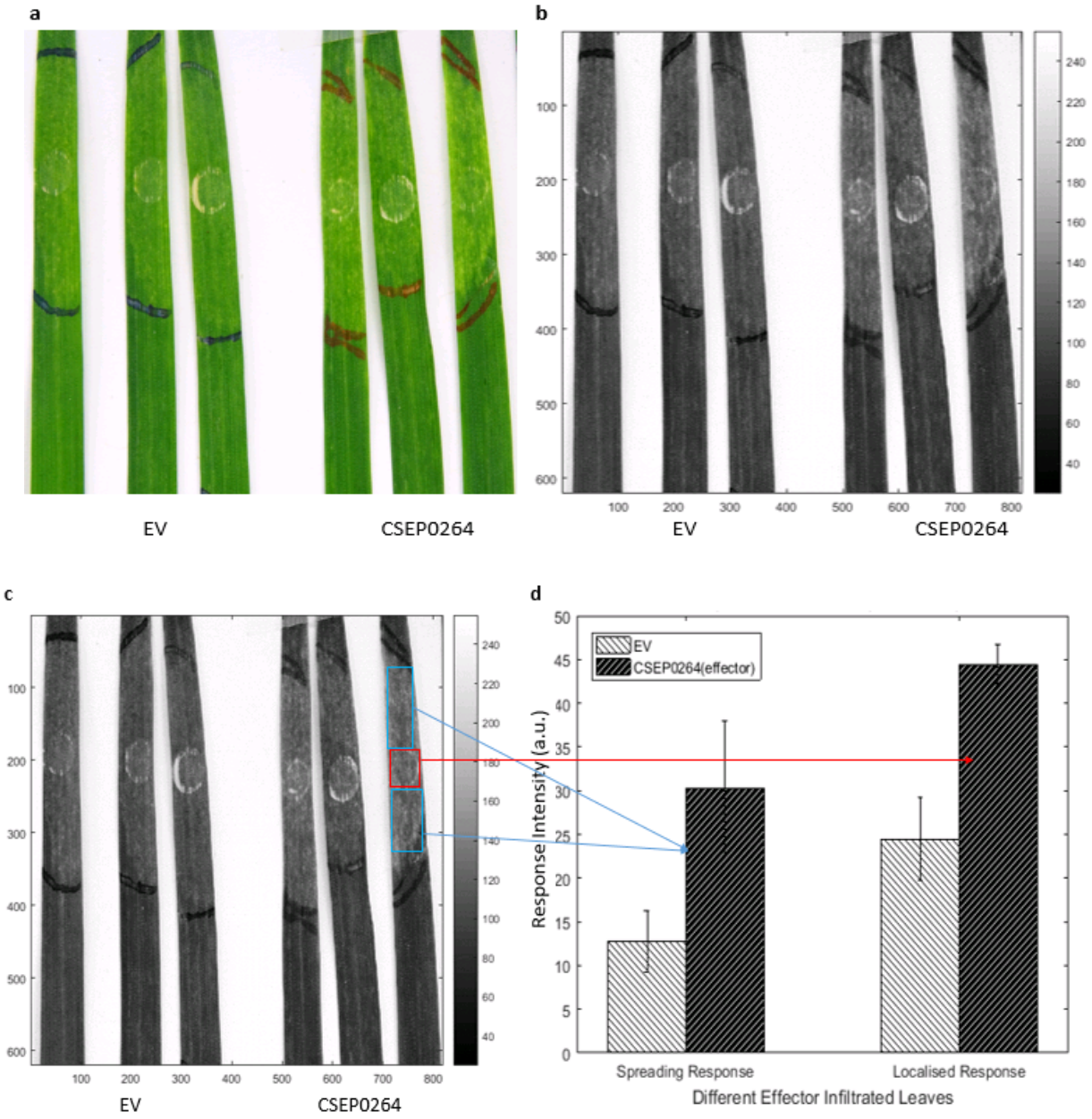

Figure 3.2 Quantification of response intensity and comparison between two treatments. (a) The original true-colour image of leaves seven days post EtHAn-infiltration. Three leaves were infiltrated with EtHAn-EV and the other three on the right with EtHAn-CSEP0264. All plants were grown, infiltrated and photographed simultaneously. (b) The grey-scale image after converting. Spreading region and localised region was selected with blue box and red box, respectively in (c). (d) There are statistically significant differences ($p < 0.05$) between EV-triggered and effector-triggered responses, in both spreading area (blue arrow) and localized region (red arrow).

3.4 Results

3.4.1 AvrPmaA1 triggers HR in wheat

An HR was readily observed by naked eyes 3 days post-infiltration on AvrPmaA1-infiltrated leaves (Figure 3.3.a). The chlorotic lesion extend to the boundaries labelled when doing the infiltration. After seven days from infiltration, the HR on AvrPmaA1-infiltrated leaves became easily recognizable as a spreading chlorosis and localized necrosis (Figure 3.3.b (1)). Leaves infiltrated with EV did not give any response except that a wound caused by syringe injection (arrow, Figure 3.3.b (2)). These indicate that EtHAn can deliver effector proteins into wheat successfully and the effector-triggered response can be screened by visual inspection in the condition used in our lab.

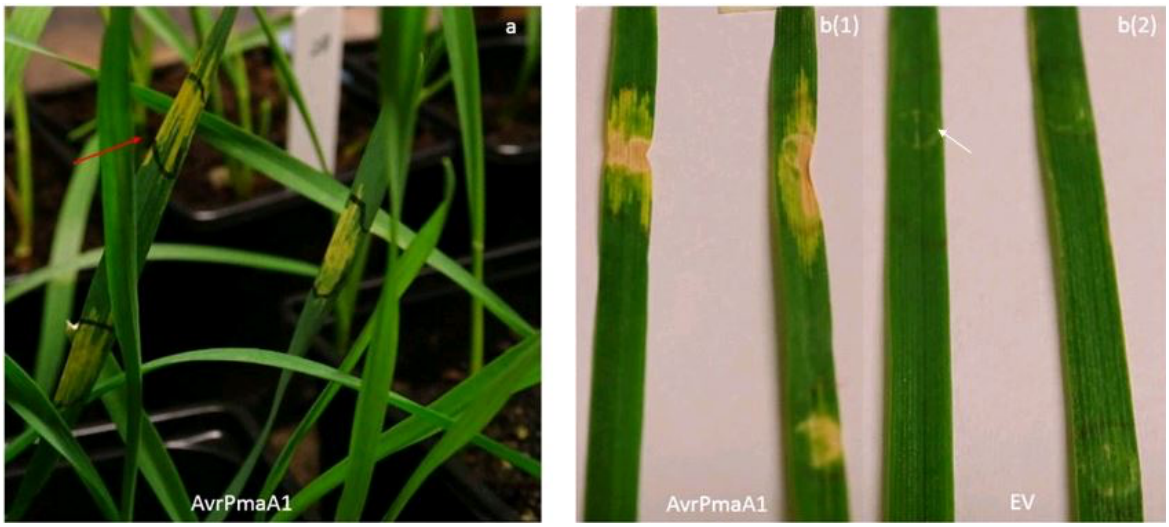

Figure 3.3 Visible responses on Cerco triggered by EtHAn-infiltration with different effectors. (a) HR (distinct chlorosis, shown by an arrow) triggered by EtHAn-deliverd AvrPma1, three days post infiltration. (b) Different phenotypes in response to EtHAn-delivered AvrPmaA1 or EV, seven days post infiltration.

3.4.2 Responses to AvrPmaA1 vary in different part of leaf

As the classification of responses triggered by EtHAn-delivered effectors were based on visual inspection, optimization of the screening system was important for better observation. After EtHAn-infiltration with AvrPmaA1 and EV at different positions of wheat (Cerco) leaves, noticeable variations in response intensity were observed (Figure 3.4). The response level triggered by EtHAn-delivered avirulence protein significantly decreased from the top to the base of leaf. More specifically, the top of leaf (2 cm and 3 cm) showed higher level of chlorosis than other positions. Again, EtHAn-EV triggered no visible response, irrespective of the position. Finally, I chose 3 cm from the tip of the leaf for all screening finally, because not only did it show the higher response intensity, but also there was enough space above it to arrow the spreading of the infiltration liquid.

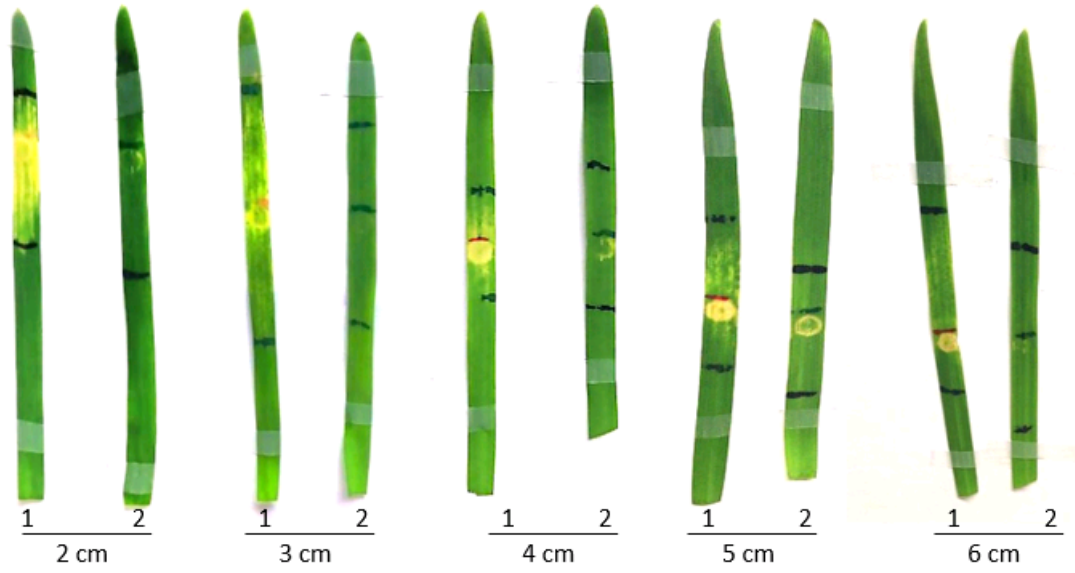

Figure 3.4 Response intensity triggered by AvrPmaA1 **varies between different parts of leaf.** All leaves were EtHAn-infiltrated and cut for photographing at the same time. Leaves labelled '1' represent leaves infiltrated with AvrPmaA1, and '2' represent EV. The avirulence protein triggered prominent chlorosis and necrosis when infiltrated at the top of the leaf (2 cm and 3 cm). The response level decreased from tip to the base of the leaf.

3.4.3 EtHAn can deliver effectors into barley

I further tested if EtHAn system can be applied to barley leaves. Unlike wheat, there is no clear HR (visible as either necrosis or chlorosis) on barley leaves that were infiltrated with AvrPmaA1. Leaves (both first and second) infiltrated with EV showed no visible response (Figure 3.5.a). HR is usually considered to be associated with reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2). The accumulation of H_2O_2 in plant cells can be a sigh of HR. DAB staining was thus carried out to detect and visualize H_2O_2 accumulation by radish-brown precipitate. Intriguingly, while no distinct chlorosis/necrosis was observed on leaves before DAB staining (Figure 3.5.a), there was a prominent brown staining which seemed more pronounced on AvrPmaA1 infiltrated area, especially on the second leaf (Figure 3.5.b), compared to the controls (leaves infiltrated with EV). This result suggested that EtHAn can also deliver effector proteins into barley leaves and trigger the immune response inside, though the phenotypic lesion was weaker and was not recognizable by simple visual inspection.

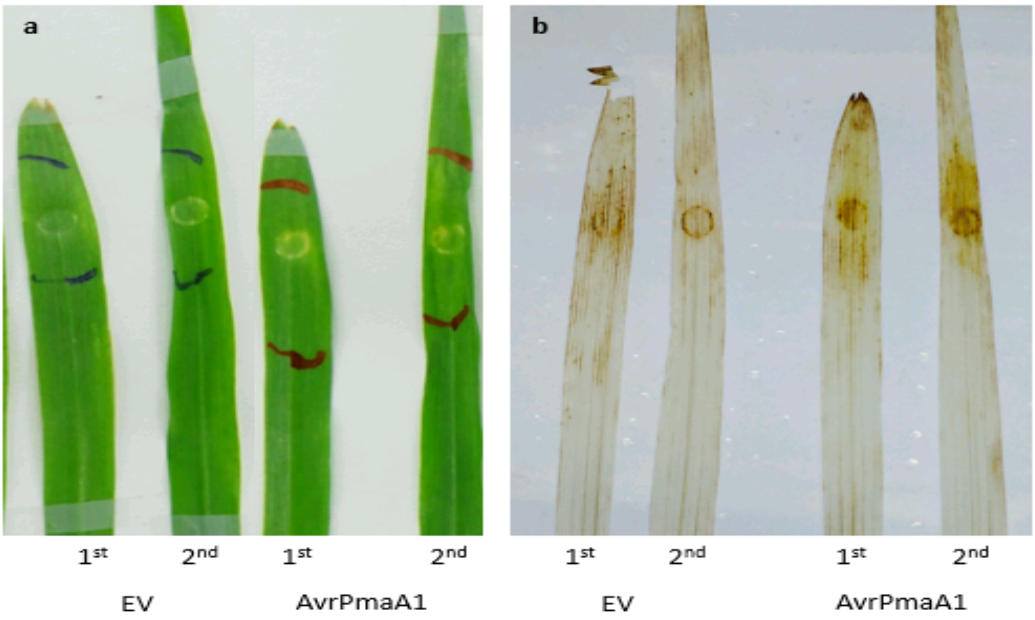

Figure 3.5 Responses on barley leaves taht EtHAn-infiltrated with different effectors before and after DAB staining. Both the first and second leaves were investigated for EtHAn-EV and EtHAn-AvrPmaA1. Four days post infiltration, leaves were photographed and then stained by DAB. (a) Four leaves before staining. Two leaves (1^{st} and 2^{nd}) infiltrated with EV were labelled with black marker pen, and two AvrPmaA1-infiltrated leaves were labelled with red marker pen. (b) The same leaves stained with DAB. The experiment was repeated once and achieved the same result.

3.4.4 Image processing pipeline

From the initial round of screening for effector-triggered response with the naked eyes, I observed that EtHAn-EV triggered response (visible as chlorosis) on some wheat lines. The EtHAn strain is derived from the soil bacterium *P. fluorescens*, which cannot infect plants. However, I observed that it can elicit PTI-like response when infiltrated into wheat. The response EtHAn-EV triggered could not be simply differentiated from that triggered by EtHAn-infiltration with other effectors (CSEP0064 and CSEP0264), which was particularly problematic (Figure 3.2). To determine if the immunity response is triggered by EtHAn-delivered effector or the bacteria itself, a more reliable approach was needed to improve the

accuracy of my screening. Thus, I developed an image processing pipeline to quantify the response intensity in an unbiased manner using MATLAB as described in 3.3.7.

3.4.5 CSEPs enhanced EtHAn-induced PTI in several lines

The workflow of large-scale screening for effector-triggered responses on 441 wheat lines is summarised in Figure 3.6. After two rounds of general screening, I confirmed that 125 wheat lines responded to EtHAn-infiltration itself. The following two rounds of screening were carried out on those 125 lines. In every single round, three treatments (EV, CSEP0064 and CSEP0264) were performed on each line. Finally the response intensity triggered by two effectors was compared with EV separately using the image processing pipeline described above. Intriguingly, two effectors triggerred significant higher ($p < 0.05$) level of chlorosis than EV on 18 lines in total. This phenomenon could be considered an enhancement of EtHAn-induced PTI by EtHAn-delivered CSEPs. CSEP0064 caused an enhancement to EtHAn-elicited PTI response on 3 lines in the first-round of screening and 5 lines in the second round. CSEP0264 elicited significant higher ($p < 0.05$) response intensity than EV on 6 lines and 4 lines in the first and second round, respectively. However, most of the 18 responding lines (except Rialto) appeared only once in two rounds of quantification and comparison. Considering the natural variations of plants, a third round of infiltration and quantification was carried out with 18 candidate lines. Taken together, lines showing significant CSEP-triggered enhancement at two or three of the total three rounds were considered ‘stable responding lines’ to this specific effector in this study.

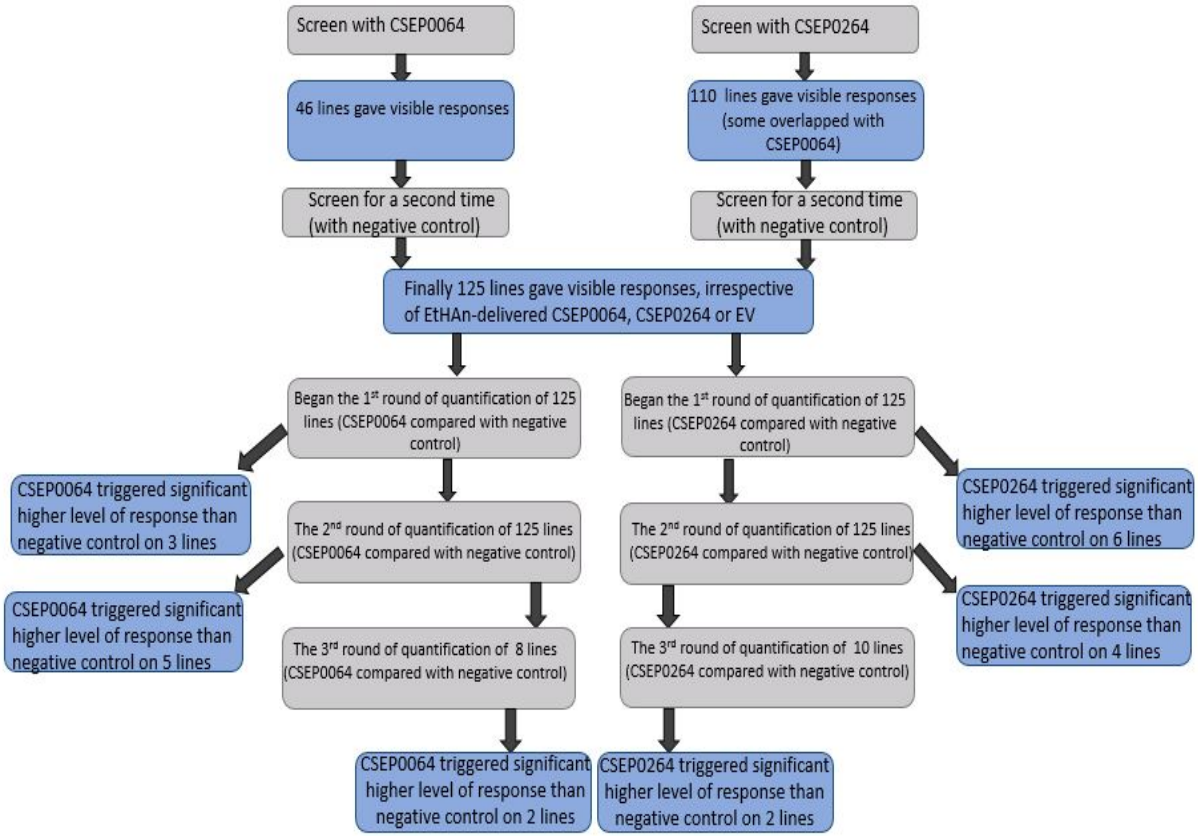

Figure 3.6 Workflow of screening for effector-triggered responses on 441 WAGTAIL wheat lines. Each round of observation/quantification was described in grey, and the results were summarised in blue text frames.

As an example, the comparison between CSEP0064 and EV on line Abbot is shown in the figure below. Clearly, CSEP0064 enhanced EtHAn-elicited PTI on line Abbot in the first and the third round (red asterisks), indicating that the chance of CSEP0064 triggering higher response intensity than EV on Abbot is 2/3. Therefore, Abbot was a line responding to CSEP0064.

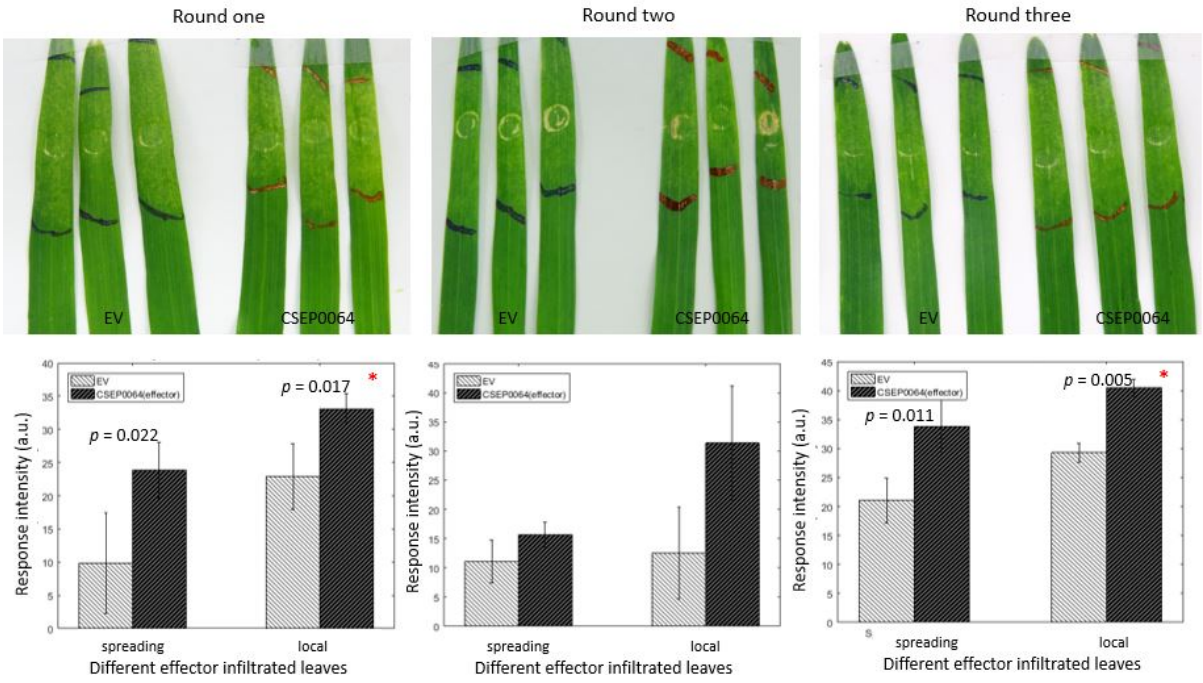

Figure 3.7 Three rounds of quantification and comparison of CSEP0064 and EV on wheat cv. Abbot. Images on the upper lane of the infiltrated leaves were from three independent experiments. Leaves labelled with red were treated with CSEP0064 and blue for EV. After quantification, the luminance of the leaves treated with CSEP0064 (black bars) and EV (grey bars) was compared for the spreading area and local area separately (described in 3.3.7). Student's *t* test was used to ascertain if the difference between two treatments was significant ($p < 0.05$). According to the *p* values calculated, CSEP0064 significantly enhanced EtHAn-triggered PTI in the first and third round (red asterisks). There was no statistically significant difference between them in round two ($p > 0.05$).

In summary, out of 441 wheat varieties screened, two lines (Abbot and Rialto) responded to delivery of CSEP0064 and two lines (Rialto and Madrigal) to delivery of CSEP0264. Therefore, only Rialto responded consistently to both effectors. The result of whole screening was summerised below with a schematic diagram (Figure 3.8). Immunity response triggered by EtHAn-infiltration was line specific (125 out of 441). There was overlap but also discrimination between two CSEPs, meaning that the additional responses triggered by effectors were both line specific and effector specific.

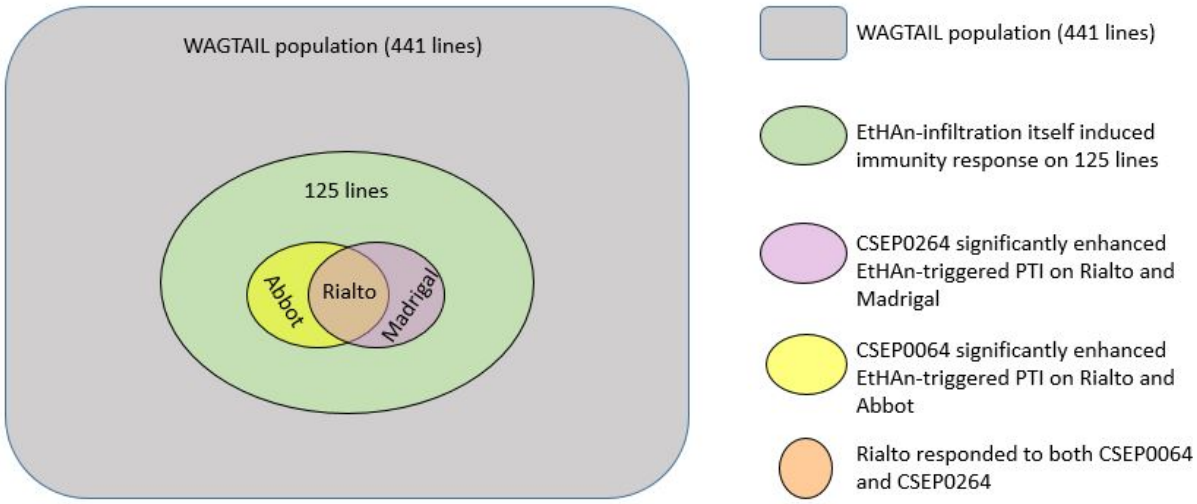

Figure 3.8 Diagram summarising the results of screening effector-triggered responses in wheat WAGTAIL mapping population. There were 125 out of total 441 lines responded to EtHAn-infiltration itself. Rialto and Abbot responded to CSEP0064, and Rialto and Madrigal responded to CSEP0264. There was one overlap between two effectors.

3.5 Discussion

One important feature of this study was the use of T3SS-based *P. fluorescens* system (EtHAn) to deliver two obligate fungal effectors (CSEP0064 and CSEP0264) into wheat and screen the responses. One bacterial avirulence protein was included as a positive control to show the typical effector-triggered responses. Different levels of AvrPmaA-triggered HR were observed when infiltration was performed at different parts of leaf, suggesting that either variations of susceptibility to EtHAn-infiltration exist in wheat or plants influence the expression of EtHAn-mediated transient system. Although it is the first time been pointed out in EtHAn system, the variations of transient expression in different leaf position have been observed in *Agrobacterium*-mediated system in many other plants including *Nicotiana* (Bashandy et al., 2015), *Arabidopsis* (Kenichi et al., 2012), lettuce and tomato (Wroblewski et al., 2005). It was suggested that the dissimilarity of leaf developmental growth causes the differences. Moreover,

leaf-development dependent differences in susceptibility to pathogen attack has been reported in monocot plants, such as barley (Le Fevre et al., 2016). Therefore, the study presented here suggests an optimized way to use EtHAn-system in wheat.

The WAGTAIL mapping population containing 441 wheat lines provided more possibilities to screen for the responses triggered by translocated effectors. The response triggered by EtHAn itself (non-pathogenic bacterium of plant) was considered to be the 'background' PTI response during the screening. It is not the first time that EtHAn was found to elicit immune response. Thomas *et al.* (2009) reported that EtHAn-infiltration induces accumulation of callose and ROS in *Nicotiana* leaves. In the following years, this was further verified on different plant species including *Arabidopsis* and wheat (Liu et al., 2016a, Upadhyaya et al., 2014). The PTI response triggered by EtHAn may come from the by-products of the T3SS machinery.

Interestingly, EtHAn selectively triggered the immune response on wheat lines. These two kinds of phenotypes of bacteria-triggered response are similar to NHR: EtHAn elicited visible chlorosis in 125 lines correspond to type II NHR response (associated with necrosis), and EtHAn elicited no visible responses in the remaining 316 lines correspond to type I NHR (does not display any visible symptoms) (Mysore and Ryu, 2004). The differentiation of two types of NHR is determined by both pathogen species and plant species (Oh et al., 2006). However, two phenotypes shown here occurred between a single pathogen species and single plant species. As a modified *P. fluorescens* strain, EtHAn is non-pathogenic to plants, thus the two types of EtHAn-elicited responses were likely to be two types of PAMP-triggered responses, rather than two types of NHR. Differentiation between PAMP-triggered defense has been previously found in a population of *Arabidopsis thaliana* with bacterial PAMPs flagellin (Vetter et al., 2016). The variation was explained by genotype specificity in their study, which can be applied to the screening system of present research as well.

Among all 125 lines that responded to the bacteria, CSEP0064 enhanced EtHAn-triggered PTI on Rialto and Abbot, and CSEP0264 enhanced the PTI on Rialto and Madrigal with one overlap. Considering that more than 400 CSEPs are shared between *B. graminis* f. sp. *hordei* and *B. graminis* f. sp. *tritici,* and homologs of barley *mildew resistance locas a* (*Mla*) have also been identified in wheat (Kusch et al., 2014, Jordan et al., 2011, Lu et al., 2016), my result may indicate that there are genes in wheat that encode the recognition and differentiation of barley powdery mildew CSEPs. But, does the recognition occur only in three lines? Given that the components and mechanisms involved underlying are still unclear, there could be different explanations for this. One possible explanation would be that EtHAn-induced PTI and effector-triggered response utilized different signalling pathways. In another word, the 'added' responses observed in those three lines were simply elicited by CSEP0064 and CSEP0264. Hence, only three lines (out of a total 441) are capable of recognizing effectors from non-adapted pathogen *B. graminis* f. sp. *hordei*. It suggests an extremely narrow distribution of wheat genes that responsible for non-host effector recognition. However, the absence of phenotypic difference between EtHAn-infiltration with CSEPs or EV does not necessarily equate with an inability to recognize effectors by the remaining lines. It is also possible that elicitation of PTI by EtHAn restricted the translocation of effectors or suppressed the effector recognition in those lines. Similar observations have been reported (Hatsugai et al., 2017). For example, the *P. syringae* pv. *tomato* DC3000-induced HR (considered to be associated with ETI) is suppressed if the same area has been previously infiltrated with non-pathogenic bacteria (Liu et al., 2016a). This possibility is based on the hypothesis that PTI commences earlier than effector recognition. However, previous studies have suggested that ETI occurs quicker and is more prolonged than PTI (Thomma et al., 2011, Robatzek, 2007). So, the non-recognition of CSEPs by other lines is unlikely to be inhibited by EtHAn-triggered PTI. It is generally accepted that ETI is the result of protein-protein interactions (Gassmann and Bhattacharjee,

2012, Giraldo et al., 2013). Accordingly, without knowing if the delivered CSEPs are recognized by R proteins in wheat, I cannot conclude without doubt that the effector-triggered response is a simple response to ETI. It has been demonstrated in various studies that the effectors can function as suppressors of PTI (Kim et al., 2009, Hauck et al., 2003, Cui et al., 2013). If this is the case for these barley powdery mildew effectors, the recognition and discrimination of two CSEPs may not be limited to three lines. Because the response of effector-infiltrated area could be a consequence of effector-triggered response compounded by a reduced PTI response.

In order to define if the effector-triggered response I observed is a consequence of ETI, additional experimental evidence will be needed. Typically, ETI is associated with HR and systemic acquired resistance (SAR) (Henry et al., 2013, De Vleesschauwer et al., 2008). Thus, investigations of these two may provide further evidence to deepen our understanding of the effector-triggered response. Taking advantage of the special colonization sites of *B. graminis*, several previous studies have successfully investigated these fungi in plants by microscopy, which can access the epidermal layer of plants (Huckelhoven et al., 1999, Huckelhoven et al., 2001, Thordal‐Christensen et al., 1997). H_2O_2 has been widely used as an indicator of HR and as well as a necessary condition of inducing SAR (Mellersh et al., 2002, Durrant and Dong, 2004). Moreover, SAR, which is a plant defense mechanism activated by previous exposure to potential pathogens, decreases the susceptibility of plants to defend themselves against latter attacks by microorganisms (Conrath, 2006, Komatsu et al., 2010). According to these, if the effector-triggered response I observed is a visible phenotype of ETI, there might be an accumulation of H_2O_2 or/and activation of SAR. Which will be further discussed in the following chapter.

Although the mechanisms that distinguish these three lines from the others are still unclear, the screening result clearly indicates that CSEP0064 and CSEP0264 are effective (visible by

significantly stronger response intensity) in eliciting plant defense responses on these three lines. There are two lines which can recognize only one of the effectors, suggesting that the genes/gene they are carrying for recognizing one effector is non-functional in recognizing the other one. The 'overlap line' Rialto may either utilize different genes to facilitate the recognition of different effectors or use same genes that capable of recognizing different effectors. An example of the latter is that the *Arabidopsis* resistance protein RPM1 recognizes two *P. syringae* effector proteins AvrB and AvrRpm1(Ashfield et al., 2014).

As CSEP0064 and CSEP0264 are two effectors from *B. graminis* f. sp. *hordei,* a non-adapted pathogen of wheat, the molecules recognizing them in wheat may also play essential roles in non-host resistance of wheat to *B. graminis* f. sp. *hordei*. However, a critical limitation of my observation is that artificially delivering effectors into non-host plants via EtHAn to trigger the immune system does not mean the same happens in NHR naturally. It is still unclear if the recognition of CSEP0064/CSEP0264 involved in the interactions between wheat and *B. graminis* f. sp. *hordei*. But the distinction of two effectors by two wheat lines still suggests that multiple and interlaced signalling pathways may involve in NHR of wheat against this pathogen.

The question why *B. graminis* f. sp. *hordei* (*B. graminis* f. sp. *tritici*) can establish a compatible relationship with barley (wheat) but not with wheat (barey) is still largely unknown. There are some similarities between these two kinds of plant resistance. For instance, H_2O_2 accumulates and papilla forms at the interaction sites between barley and *B. graminis* f. sp. *hordei*, but also between barley and *B. graminis* f. sp. *tritici* (Huckelhoven et al., 1999). Another study showed that inoculating barley with three different pathogens: *B. graminis* f. sp. *hordei*, *B. graminis* f. sp. *tritici* and *P. syringae* leads to the up-regulation of defense-related genes including *PR1, PR3, PR5* and *PR9* (Vallélian-Bindschedler et al., 1998). More recently, PR5 is identified as one of the host proteins that interact with CSEP0064 in barley (Pennington

et al., 2016). Beyond powdery mildews and cereals, the similarities between HR and NHR have been investigated in various pathogen-plant interactions (Hatsugai et al., 2017, Gill et al., 2015). In the context of this study, another important observation is that EtHAn can also deliver effector proteins into barley plants. Even though the expression of the bacterial avirulence protein in barley did not lead to visible symptoms on the leaf surface, the H_2O_2 accumulation detected by DAB provided substantial evidence of the recognition of translocated AvrPmaA1 inside. This suggests a broader application of EtHAn system. Notably, it provides a possibility of investigating introduced CSEPs in host plant barley and non-host plant wheat simultaneously.

3.6 Conclusions

The *P. fluorescens*-based EtHAn system can be used to deliver barley powdery mildew effectors into the non-host plant wheat. Although the bacterium itself triggered PTI in wheat, three wheat cultivars (Rialto, Abbot and Madrigal) from WAGTAIL population were capable of consistently recognizing and differentiating introduced CSEP0064 or CSEP0264, leading to an enhancement of EtHAn-triggered PTI. Moreover, the effector-triggered responses can be screened by visual inspection and quantified using the image-processing pipeline I developed.

3.7 Future work

Although several WAGTAIL lines were identified during screening that may carry the gene/genes to facilitate the effector recognition, the number of responding lines is too small to carry out any meaningful mapping in the WAGTAIL mapping population. Therefore, the same screening for CSEP-triggered responses could be performed on another wheat mapping population MAGIC. The advantages of using MAGIC population include that: first, it was created by intercrossing eight parental lines for several generations to accumulate the recombination events and improve the mapping precision; second, Rialto is one of the eight

founders; third, it is better demonstrated and more widely used in other studies (Gardner et al., 2016). Thus, MAGIC will be a logical population for mapping to identify the recognition allele of CSEPS.

4 EtHAn-delivered CSEPs trigger systemic changes in susceptibility to powdery mildew, in wheat

4.1 Introduction

To protect themselves from invading pathogens, plants are armed with constitutive and inducible defences (Durrant and Dong, 2004, Kogel and Langen, 2005). As described in Chapter 3, plants use PTI and ETI to alleviate pathogen attacks locally (Jones et al., 2006, Taguchi et al., 2010). Moreover, the induction of local responses can induce signals that lead to resistance systemically (Balmer et al., 2013b). Induced systemic defense responses in plants can be broadly divided into two forms, systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Figure 4.1) (Liu et al., 2010b, Choudhary et al., 2007). SAR can be triggered when plants encounter virulent and avirulent pathogens (Aryal et al., 2011), whereas ISR is generally activated by colonization of non-pathogenic rhizobacteria (Weller et al., 2012, Attaran et al., 2009).

SAR is a phenomenon whereby prior (primary) infection or treatment renders uninfected systemic (distal) tissues more resistant against subsequent infections (Fu and Dong, 2013). SAR is long-lasting and can persist for up to month (Shah and Zeier, 2013); it also confers resistance to a broad range of subsequent infections (De Vleesschauwer et al., 2008, Savatin et al., 2014). Although typical SAR is triggered by ETI associated with programmed cell death (Kiefer and Slusarenko, 2003, Selin et al., 2016), it has been reported that cell death is dispensable for ETI-mediated SAR (Zhang and Zhou, 2010). For example, recognition of Potato Virus X (PVX) by *Rx* gene induces SAR to later tobacco mosaic virus (TMV) infection in *Nicotiana* in the absence of cell death (Liu et al., 2010b). In addition to ETI, PTI has the

potential to elicit SAR as well (Figure 4.1) (Aryal et al., 2011, Boller and Felix, 2009). Two PAMPs, flagellin and lipopolysaccharides from non-adapted *P. syringae* strains induce SAR in *Arabidopsis* (Mishina and Zeier, 2007). Moreover, infiltration of *Arabidopsis* leaves with *Bacillus cereus* also activates PTI and induced SAR (Niu et al., 2016).

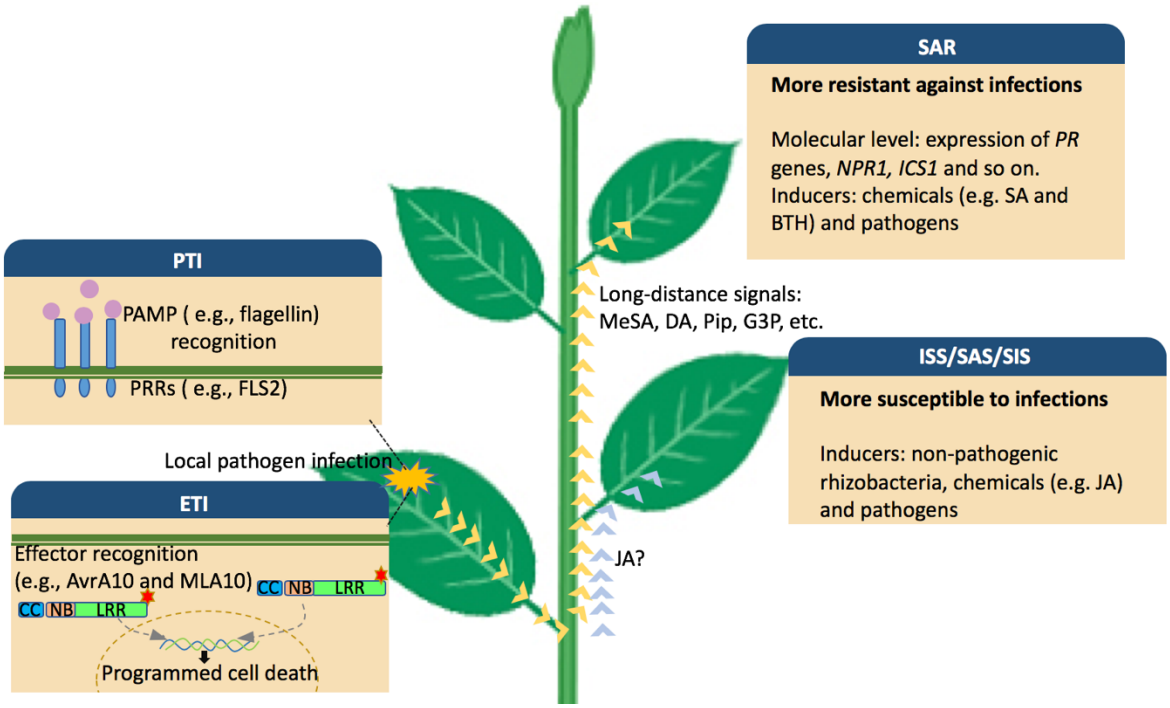

Figure 4.1 Scheme of induced immunity in plants. Plant cell wall and membrane: thick and thin green lines. PAMP: light purple circle. Effector: red star. Plants use PTI and ETI to alleviate pathogen attacks locally. The induction of local responses can induce signals that lead to systemic resistance or susceptibility. Some signals (yellow route) that play a role in SAR have been identified, while the signalling pathway of ISS (blue route) is still unclear. See details in discussion. Adapted from Balmer et al. (2013).

In the last few decades, many studies have proved that plant defense hormone salicylic acid (SA) plays a pivotal role in SAR (Boatwright and Pajerowska-Mukhtar, 2013, Chigurupati et al., 2016). For instance, loss of SAR in *Arabidopsis* by knocking out a flavin-dependent monooxygenase (*FMO1*) gene is accompanied by the failure to initiate SA accumulation (Mishina and Zeier, 2006), SAR is compromised in transgenic *Arabidopsis* plants that express

the *NahG* gene, which encodes salicylate hydroxylase to metabolize SA (Lawton et al., 1995); furthermore, application of SA or its functional analogs benzothiadiazole S-methyl ester (BTH) and 2,6-dichloroisonicotinic acid (INA) can also induce SAR in *Nicotiana* and *Arabidopsis* plants (Chern et al., 2008, Cortes‐Barco et al., 2010). Although the establishment of SAR requires mobile signals to facilitate the long-distance communications between infected/treated and distant plant tissues, SA itself is unlikely to travel from local to systemic tissues during SAR. Several studies have provided experimental evidence to support this. As an example, application of BTH on cucumber triggers SAR without accumulation of SA in systemic leaves (Liu et al., 2008). Additionally, SAR develops in *Arabidopsis* after inoculation with *P. syringae* and at the same time SA biosynthesis gene isochrismate synthase 1(*ICS1*) is significantly upregulated in systemic leaves, suggesting that SA accumulation there is due to *de novo* synthesis rather than translocation (Attaran et al., 2009). These lead to the requirement for identifying candidate long-distance signals in SAR (Figure 4.1). Some of such factors have been found in different plants, including methyl salicylate (MeSA) in potato (Manosalva et al., 2010), dehydroabietinal (DA) in *Arabidopsis* (Chaturvedi et al., 2012), pipecolic acid (Pip) in *Arabidopsis* (Bernsdorff et al., 2016), glycerol-3-phophate (G3P) in *Nicotiana* (Chanda et al., 2011). These factors may be dependent, partially dependent or independent of SA (Gruner et al., 2013, Wittek et al., 2014). In the SA-mediated signalling pathway, a key component is the transcriptional co-activator non-expressor of pathogenesis-related genes 1 (*NPR1),* which is considered to be a receptor of SA (Fu et al., 2012). Activation of *NPR1* and its paralogs *NPR3* and *NPR4* are usually associated with induced expression of *PR* genes, for example *PR1* and *PR5* (Boatwright and Pajerowska-Mukhtar, 2013, Gao et al., 2015). Therefore, activation of *PR* genes is commonly considered to be a marker during SA-mediated SAR (Caarls et al., 2015).

To date, the vast majority of knowledge about SAR is gathered from dicots. Compared to the progress in understanding SAR in dicots, SAR in monocots is still poorly understood. Nevertheless, some factors that play a role in SAR in dicots, have also been investigated in monocots. Over-expression of *Arabidopsis NPR1* gene in rice enhances the resistance of transgenic plant against *Xanthomonas oryzae* pv. oryzae (Chern et al., 2001) and knockdown of the *NPR1* ortholog in rice compromises the BTH-induced rice resistance to *Xanthomonas oryzae* pv. oryzae (Sugano et al., 2010), suggesting the involvement and importance of *NPR1* orthologs in SA-mediated signalling in rice. Another study on rice demonstrated that transgenic plants expressing *NahG* (encodes the SA-degrading enzyme) exhibit a reduced resistance against the rice blast fungus (Yang et al., 2004). In addition to factors involved in SA-mediated signalling, chemical inducers of SAR active in dicots have also been investigated in cereals. For example, application of BTH on wheat plants at the jointing stage significantly increases the concentration of phenolic compounds in wheat grains (Ramos et al., 2017). Phenolics were chosen in their study because they were found to act during SAR development in *Arabidopsis* (Alves et al., 2013, Voigt, 2014). *PR* genes, are other key players of SAR in dicots. Interestingly, treatment with BTH on maize seeds triggers the expression of *PR1* and *PR5* in maize leaves (Balmer et al., 2013a). Moreover, inoculation of barley leaves with non-adapted *P. syringae* results in increased resistance in the adjacent area to *M. oryzae* and adapted *P. syringae* strain (Colebrook et al., 2012). The transcripts associated with the adjacent resistance are similar to that of SAR in *Arabidopsis*.

Although these results indicate that many SAR players conserve in dicots and monocots, only a few recent reports described the SAR or SAR-like phenomenon in monocots. For example, a protein elicitor-encoding gene (*PemG1*)enhances the resistance of rice against the subsequent bacterial infection systemically and this is accompanied by expression of *PR* genes, the accumulation of H_2O_2 and over-expression of SA signal-related genes in rice (Peng et al.,

2011). Besides, primary infection with *P. syringae* or *X. translucens* induce resistance in uninfected barley leaves against a second challenge with *X. translucens* (Dey et al., 2014). A more recent study showed that chitin treatment of the first leaf enhances resistance to *B. graminis* f. sp. *hordei* in the second (untreated) leaf of barley (Kasbauer et al., 2018). In maize, leaf infection with *C. graminicola* elevates the expression level of SA in systemic untreated leaves and triggers SAR against the subsequent infection from the same pathogen (Balmer et al., 2013a). In the context of wheat, inoculation of the fourth (local) leaves with an incompatible strain of *Puccinia striiformis* f. sp. *tritici* enhances the resistance against a compatible *Puccinia striiformis* f. sp. *tritici* strain in the fifth (systemic) leaves (Yang et al., 2013).

It is worth noting that primary infection can confer enhanced susceptibility to subsequent attackers in plants (Figure 4.1). Although the systemically induced susceptibility in plants has not been conclusively elucidated, a few studies have reported such phenomenon. Pre-treatment with jasmonic acid (JA) on the first leaves renders the second leaves of cucumber more susceptible to *Colletotrichum orbiculare* (Liu et al., 2008). They name this phenomenon as systemic acquired susceptibility (SAS). Inoculation with *P. fluorescens* on roots of *Arabidopsis* compromises the resistance against aphid *Myzus persicae* in leaves (Pineda et al., 2012). The root-to-leaf signalling which acts in the opposite way to ISR presented in that study is called induced systemic susceptibility (ISS). Another example is that primary infection of *Arabidopsis* lower rosette leaves with *P. syringae* enhances susceptibility of the upper leaves to moth *Trichoplusia ni,* where the response is concluded as systemic induced susceptibility (SIS) (Groen et al., 2013). Additionally, the induced susceptibility at systemic level has also been described in jack pine (Klutsch et al., 2017) and pepper plants (Garcia et al., 2015). Unlike SAR in which SA-mediated signalling plays a vital role, systemic induced/acquired susceptibility is suggested to involve JA-mediated signalling (Liu et al., 2008) or antagonistic

signalling between SA and JA (Haney et al., 2017, Caarls et al., 2015). To my knowledge, there has been no report that demonstrates the induced susceptibility in monocots at a systemic level.

In Chapter 3, the bacteria and effector-triggered responses are described, which shown as local chlorotic/necrotic lesions in wheat. In this chapter, I investigate if the recognition of bacteria/delivered effectors can alter the immune ability of wheat locally/systemically. Also, it is tested if the differential responses to the effectors in different wheat cultivars results in different systemic responses.

4.2 Objectives

1. To investigate if the recognition of EtHAn-delivered CSEPs in wheat can enhance resistance/susceptibility locally to subsequent fungal infection.

2. To show if the CSEPs can influence the resistance/susceptibility systemically in wheat.

3. To test changes in immunity to non-adapted *B. graminis* f. sp. *hordei* or adapted *B. graminis* f. sp. *tritici*.

4.3 Materials and Methods

4.3.1 Plant and fungal materials

Five wheat cultivars including Rialto, Abbot, Madrigal, Hardi and Cerco were grown as described in Chapter 3. Barley (*H. vulgare*) cv. Golden Promise seedlings were grown in a 16-hour light/8-hour dark cycle at 20 ℃. *B. graminis* f. sp. *hordei* was maintained on living barley plants in a dedicated enclosed transparent container protected from air currents that may displace the conidia. Old conidia were removed by shaking the inoculum pots one day before infecting healthy plants. New fungal stocks were made every week by inoculating seven-day seedlings with *B. graminis* f. sp. *hordei. B. graminis* f. sp. *tritici* was maintained in a different growth room on wheat (*Triticum aestivum*) cv. Cerco plants in the same way.

4.3.2 Wheat immunity assay design

The first leaves of different wheat lines were infiltrated with EtHAn as described in Chapter 3. Seven days after the infiltration when the response phenotype had developed, the whole plants with fully expanded second leaves were transferred to inoculation chambers for *B. graminis* f. sp. *hordei* or *B. graminis* f. sp. *tritici*. The plants were then inoculated with *B. graminis* f. sp. *hordei/B. graminis* f. sp. *tritici* and kept in a dedicated enclosed transparent growth chamber. After two days, the second leaves were collected to assess the systemic responses to fungal infection and the first leaves were harvested for investigating the local responses. Determination of the plant defense responses was based on the observation of fungal structure development. Fungal structures were stained, then newly-established colonies and spores at different stages were counted. The percentage of colony/total conidia on each leaf was used to determine the possibility of successful fungal colonization. The higher the value, the more susceptible the leaf.

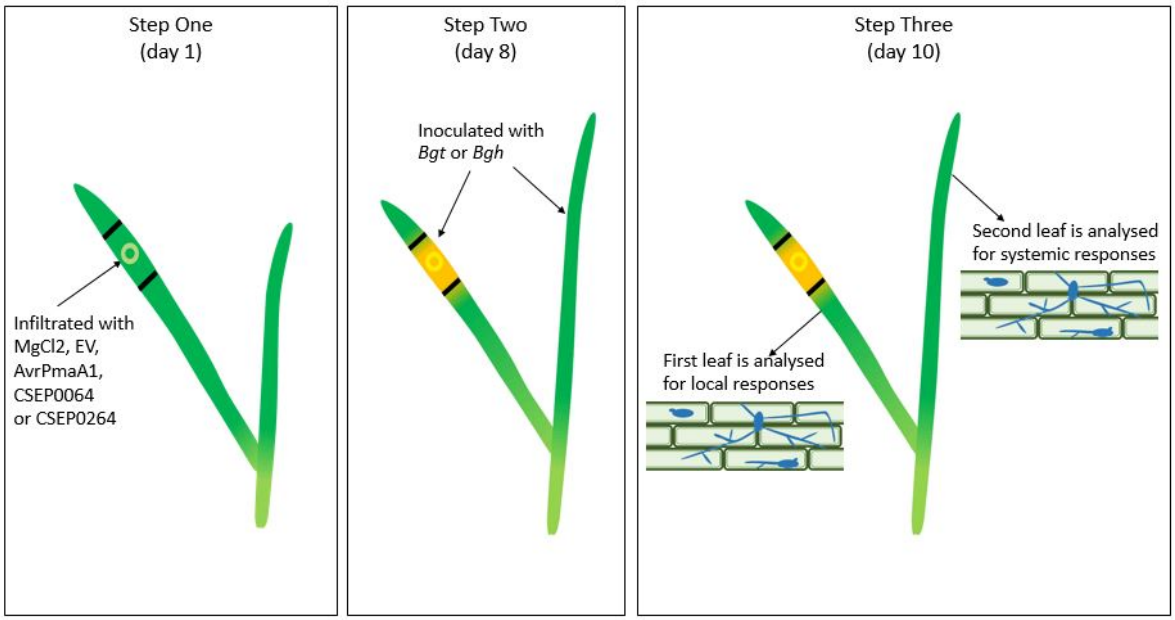

Figure 4.2 Workflow for EtHAn-based wheat immunity assay. The first leaf was EtHAn-infiltrated (step one) to induce local defense responses. The plants were subsequently inoculated with *B. graminis* f. sp. *hordei/B. graminis* f. sp. *tritici* seven days after infiltration (step two).

Two days later, the fungal development level was examined to reveal the plant susceptibility both locally and systemically (step three).

4.3.3 Lactophenol Cotton Blue staining and microscopy

Lactophenol Cotton Blue (Pro-Lab Diagnostics, USA) was used to stain fungal structures. Three detached infected leaves were immersed in staining solution (Lactophenol Cotton Blue: ethanol = 1:2 (v/v)) in a 15 ml tube. The tube was then placed in a heated water bath at 87 °C for 90 seconds. The leaves were left overnight in staining solution at room temperature. Destaining was carried out the next day. The staining solution was first discarded into a phenol waste bottle and leaves were covered with destaining solution (acetic acid: ethanol = 1:3 (v/v)). After gentle shaking, destaining solution was transferred to the phenol waste bottle. Then the tube was filled with destaining buffer again and left overnight. The tissues can be kept in destaining solution for several days before microscopy.

Every destained single leaf was cut into three fragments and placed onto a microscope slide with the abaxial side facing upwards. Bright-field microscopy was performed using a compound microscope (ZEISS, Germany).

4.3.4 Statistical analysis

The number of plants and biological/ technical replicates are listed below. For each treatment on each wheat line, the mean and standard error were calculated. For each wheat cultivar, the percentage of successful colony formation on plants that pre-infiltrated with AvrPmaA1/ CSEP0064/ CSEP0264 was compared to that on plants pre-infiltrated with an 'empty vector' (EV) control. Student's *t*-test was used to determine whether there was a significant difference between the means of two treatments. $p < 0.05$ was considered to be statistically significant in this study.

Table 4.1 Number of plants included to test the local and systemic susceptibility to *B. graminis* f. sp. *tritici* after infiltration with different effectors. The susceptibility of wheat to *B. graminis* f. sp. *hordei* was tested using the same number of plants as listed below.

Wheat variety	Plants	Rounds	Independent experiments	Total plants
Rialto	3	3	3	27
Abbot	4	3	2	24
Madrigal	4	3	1	12
Hardi	4	3	1	12
Cerco	3	3	3	27

4.4 Individual contributions

I performed the susceptibility test for six independent experiments on Rialto (total 18 samples), all experiments on Abbot and Cerco, two independent experiments on Madrigal (6 samples).

Stacey Vincent, a MRes student I supervised, performed another three independent experiments on Rialto to see if my results can be reproduced. She also did the others replicates for Madrigal and Hardi.

4.5 Results

4.5.1 B. graminis *f. sp.* tritici *or* B. graminis *f. sp.* hordei *development on wheat leaves*

Microscopy of inoculated wheat leaves (2 dpi) showed the development of *B. graminis* f. sp. *tritici* and *B. graminis* f. sp. *hordei*. Almost all spores geminated and formed appressoria. Some *B. graminis* f. sp. *tritici* spores breached the plant cell wall successfully and developed colonies with feeding structures haustoria (Figure 4.3.a). Occasionally, *B. graminis* f. sp. *hordei*

sporelings showed successful penetration, which was indicated by the presence of haustorium and colony (Figure 4.3.b). Although short hyphae were produced, further development of *B. graminis* f. sp. *hordei* invariably stopped (Figure 4.3.d). Conversely, many *B. graminis* f. sp. *tritici* hypae proliferated abundantly in the following few days (Figure 4.3.c), which made it difficult to determine the quantities. Therefore, I chose to count the colonies and conidiospores two days after inoculation. For example in Figure 4.3.b, the quantities of total spores and colonies were five and one, respectively. Therefore, the colonization efficiency would be 20%.

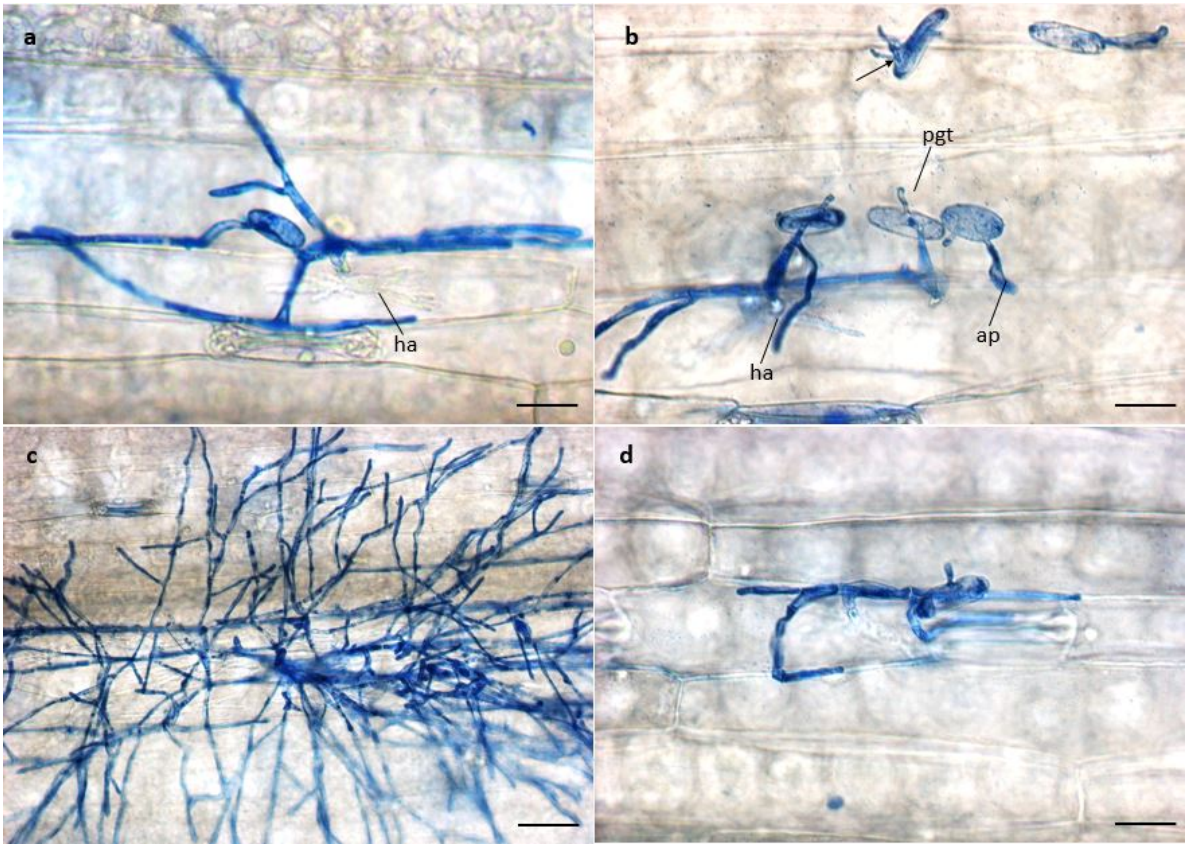

Figure 4.3 Microscopy of wheat/barley powdery mildew developments on wheat cv. Cerco leaves. External fungal structures were stained using Lactophenol Cotton Blue. ha, haustorium; ap, appressorium; pgt, primary germ tube. (a) A *B. graminis* f. sp. *tritici* sporeling penetrated into the epidermal cell and formed a multidigitated haustorium. A new colony developed at the successful penetration site at 2 dpi. Bar = 20 µm. (b) Most of the landed *B. graminis* f. sp. *hordei* spores germinated but failed to breach the cell wall. Rarely, a spore showed multiple attempts as indicated by multiple germ tubes (arrow). Very occasionally, a *B. graminis* f. sp. *hordei* spore

can penetrate into cell wall, a colony associated with haustorium formed on wheat at 2 dpi. Bar = 20 µm. (c) *B. graminis* f. sp. *tritici* produced masses of colonies at 5 dpi. Bar = 80 µm. (d) Unlike *B. graminis* f. sp. *tritici*, any *B. graminis* f. sp. *hordei* hyphae that started growing on wheat, stopped without any further development. Bar = 20 µm.

4.5.2 EtHAn-infiltrated effectors regulated wheat resistance/susceptibility against B. graminis *f. sp.* tritici

To test the effects of EtHAn infiltration on the susceptibility of wheat against the adapted fungus *B. graminis* f. sp. *tritici*, five different treatments were applied on five wheat varieties. Infiltration with $MgCl_2$ was first compared to un-infiltrated plants to see if the wounding caused by syringe infiltration affect the immune system of wheat. Both events showed nearly same level of resistance to *B. graminis* f. sp. *tritici* (data not shown here). Therefore, $MgCl_2$ was used as a negative control to show the basal level of resistance plants possessed. Given that effector (AvrPmaA1, CSEP0064 or CSEP0264) triggered responses were always associated with an EtHAn-induced PTI, EV was also used as a control to determine if the induced susceptibility change is due to recognition of specific effectors or of the bacterium itself.

4.5.2.1 AvrPmaA1 *and CSEPs enhanced systemic disease resistance in cv. Rialto*

After screening of the WAGTAIL wheat mapping population, Rialto responded to both CSEP0064 and CSEP0264 (Chapter 3). On inoculated Rialto plants, similar frequencies of *B. graminis* f. sp. *tritici* colonization were observed on plants that had been infiltrated with $MgCl_2$ or EV (Figure 4.4), suggesting that EtHAn-triggered PTI did not affect the defense mechanisms of wheat to subsequent *B. graminis* f. sp. *tritici* infection. On average, about 10% of the spores developed colonies on EV-infiltrated first leaves; on second leaves, fewer 5% spores formed colonies. There was no significant difference between all treatments on the first leaves (Figure 4.4.a), whereas only a very small proportion of spores penetrated and established colonies on the second leaves after EtHAn-infiltration with effectors (Figure 4.4.b). Thus, infiltration with

AvaPmaA1, CSEP0064 and CSEP0264 reduced the colony development frequency on second leaves to 0.09%, 0.48% and 0.16%, respectively. The dramatic decrease compared to EV control suggested that recognition of three delivered effectors in local leaves induced wheat resistance to *B. graminis* f. sp. *tritici* systemically.

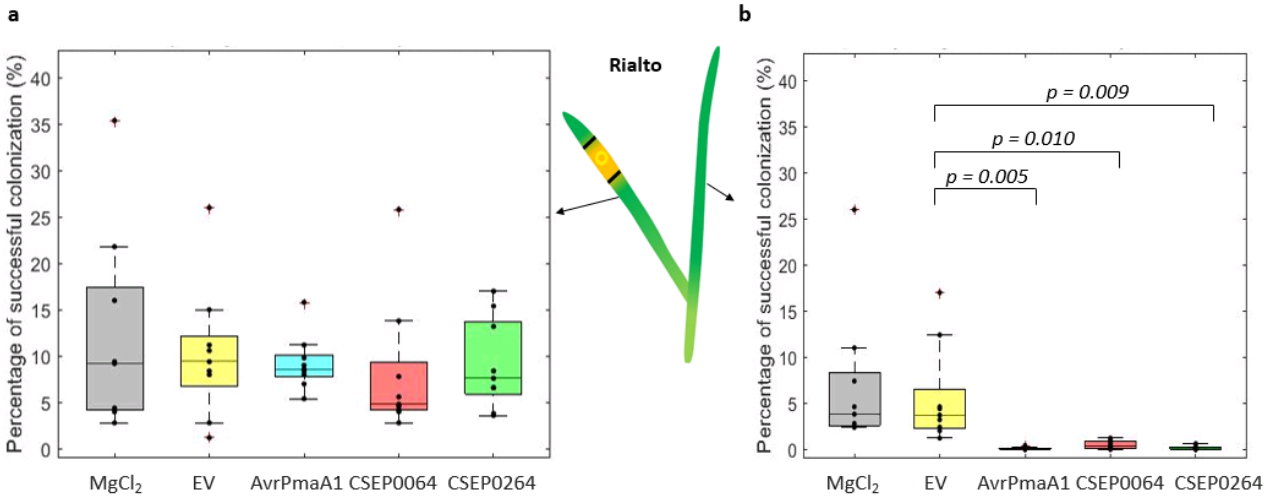

Figure 4.4 Effects of EtHAn-infiltration with different effectors on local/systemic defense resistance of Rialto. The first leaves of Rialto were infiltrated with $MgCl_2$, EV, AvrPmaA1, CSEP0064 or CSEP0264 separately. After 7 days, the whole plants were challenged by *B. graminis* f. sp. *tritici*. Colonies and spores were counted for both first leaves and second (systemic) leaves. Box plots for n = 27 samples (nine independent experiments) with whiskers extending to a maximum of 1.5 x IQR. Each single dot shown was the mean of three biological replicates. The difference between different effectors and EV was defined by Student's *t*–test. $p < 0.05$ was considered to be significant. (a) Susceptibility of first leaves to *B. graminis* f. sp. *tritici*. There was no statistically significant difference between all treatments. (b) Susceptibility of second leaves to *B. graminis* f. sp. *tritici*. Reduction in colony formation percentage showed in plants that infiltrated with avirulence protein, CSEP0064 or CSEP0264. The differences between three effectors and EV were highly significant with *p* values of 0.005, 0.010 and 0.009, respectively.

To further investigate the effector-triggered systemic resistance in Rialto, I investigated when it happened and how long the effects persist. I performed a time-course of EtHAn-

infiltration before subsequent *B. graminis* f. sp. *tritici* inoculation. Infiltrated plants were inoculated with *B. graminis* f. sp. *tritici* after 0 day (immediately), 4 days, 7 days, 10 days and 14 days. Then the fungal development on second leaves was observed and compared between EV and effectors for each time point. Generally speaking, the plants became more resistant to fungal infection over the time, this was indicated by decreased levels of successful colonization (Figure 4.5). AvrPmaA1 and two CSEPs were not able to show sufficient effects on enhancing systemic resistance if the plants were inoculated with *B. graminis* f. sp. *tritici* within four days from infiltration. However, the effector-induced systemic resistance reached a significant level in seven days from effector delivery. Moreover, the systemic resistance triggered in effector-treated plants persisted for at least 14 days post infiltration.

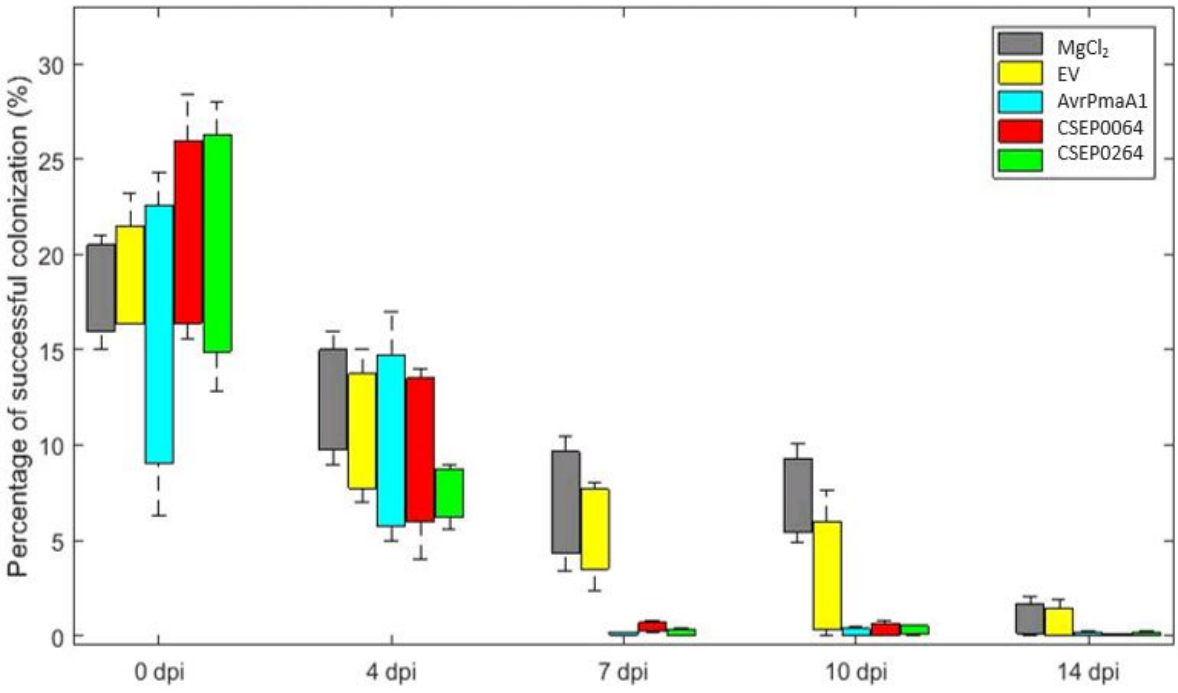

Figure 4.5 Susceptibility of second leaves across a time-course of EtHAn-infiltration before *B. graminis* f. sp. *tritici* infection. Plants were infiltrated first, then inoculated with *B. graminis* f. sp. *tritici* at 0 day post infiltration (dpi), 4 dpi, 7 dpi, 10 dpi and 14 dpi. There was no obvious difference between effector-treated and EV-treated plants if the plants were inoculated within four days since infiltration. From 7 dpi till 14 dpi, fungal development was

largely inhibited by EtHAn-infiltrated AvrPmaA1, CSEP0064 and CSEP0264. The plants became more resistant to *B. graminis* f. sp. *tritici* infection over the time-course.

4.5.2.2 AvrPmaA1 *and CSEPs triggered systemic susceptibility in cv. Abbot*

I next tested local and systemic defense resistance against *B. graminis* f. sp. *tritici* in cv. Abbot, which responded to AvrPmaA1 and CSEP0064 (i.e. showed higher level of chlorosis than EV). I compared the levels of susceptibility to fungal development on first leaves that were infiltrated with EV and different effectors, the difference between them was not obvious (Figure 4.6.a). On non-infiltrated systemic leaves, approximately two percent of the spores colonized successfully the EV and $MgCl_2$ controls. Intriguingly, the *B. graminis* f. sp. *tritici* sporelings were more likely to penetrate and establish colonies on the systemic leaves of plants that had been pre-treated with effectors. The ratio of successful colonization increased to 5.0%, 5.7% and 4.4% after prior infiltration with AvrPmaA1, CSEP0064 and CSEP0264, respectively (Figure 4.6.b). Therefore, EtHAn-infiltration with three effectors resulted in enhanced susceptibility to *B. graminis* f. sp. *tritici* in Abbot. The enhancement of susceptibility was at a systemic level rather than locally.

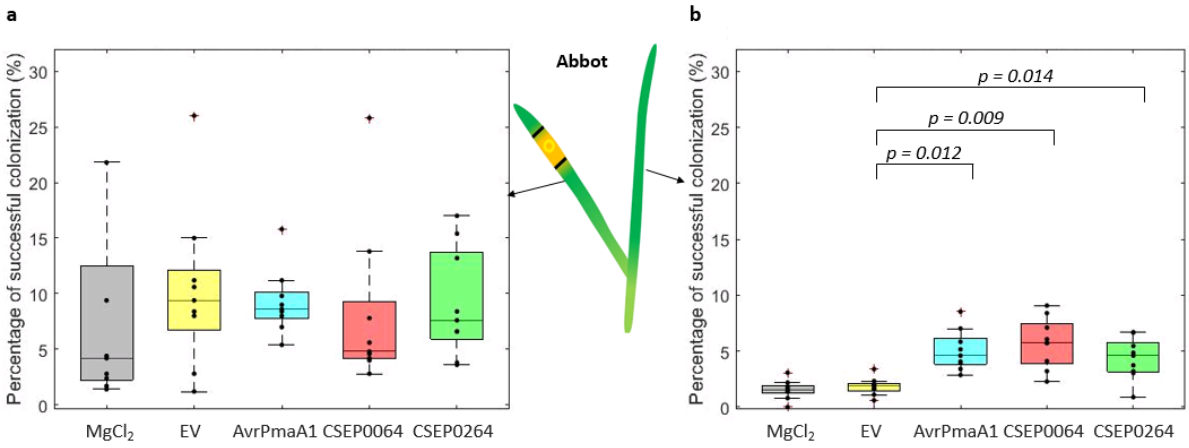

Figure 4.6 Effects of EtHAn-infiltration with different effectors on local/systemic susceptibility to *B. graminis* f. sp. *tritici* in Abbot. Data was collected from n = 24 samples from eight independent experiments. Each single dot shown was the mean of three biological replicates. Percentages between different effectors and EV were compared and the difference was defined by Student's *t*–test. $p < 0.05$ was considered to be significant. (a) Fungal development on first leaves. No significant difference was observed between all treatments. (b) AvrPmaA1, CSEP0064 and CSEP0264 strikingly promoted fungal colonization than EV on the systemic second leaves. The differences between them were statistically significant, indicated by $p = 0.012$, $p = 0.009$, $p = 0.014$.

4.5.2.3 AvrPmaA1 *and CSEP0064 induced systemic resistance in cv. Madrigal*

Madrigal was a cultivar which responded to AvrPmaA1 and CSEP0264. CSEP0064 did not enhance EtHAn-triggered chlorosis on Madrigal. When I inoculated these plants with *B. graminis* f. sp. *tritici*, I observed that infiltration with AvrPmaA1 and CSEP0064 resulted in less fungal colonization on second leaves. Compared to EV control, on which 3.9% of total spores developed into colonies, AvrPmaA1 and CSEP0064 induced a decrease of the frequency to 1.0% and 1.5%, respectively (Figure 4.7.b). Although the frequency of successful colonization appeared lower on CSEP0264 treated plants (second leaves) as well, this was not significantly different from that of EV; note, though, that on these plants the spread of the result data was much greater. All treatments showed similar levels of resistance to *B. graminis* f. sp.

tritici on the first leaves of Madrigal (Figure 4.7.a). Taken together, EtHAn-infiltration with CSEP0064 and AvrPmaA1 induced resistance against *B. graminis* f. sp. *tritici* infection systemically.

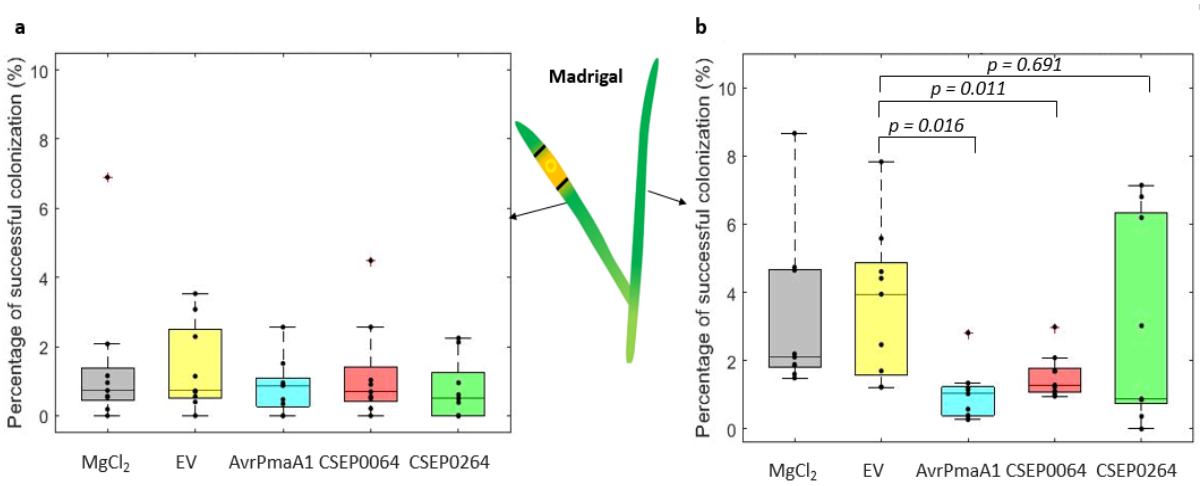

Figure 4.7 Effects of EtHAn-infiltration on local and systemic resistance to *B. graminis* f. sp. *tritici* in Madrigal. Box plots for n = 12 samples (leaves). (a) Similar proportions of spores developed colonies on first leaves that had been infiltrated with $MgCl_2$/EV controls or different effectors. (b) In comparison with EV, AvrPmaA1 and CSEP0064 significantly inhibited colony development on second leaves, shown by $p = 0.016$ and $p = 0.011$, respectively. CSEP0264 showed a lower average of colony formation percentage than EV, but without statistically significant difference due to huge variations among samples.

4.5.2.4 AvrPmaA1 *and CSEP0064 enhanced local susceptibility to* B. graminis *f. sp.* tritici *in cv. Cerco*

EtHAn-infiltration with AvrPmaA1 triggered chlorotic responses on Cerco plants. But there was no visible responses triggered by CSEP0064 or CSEP0264 on this line. After inoculating pre-infiltrated Cerco plants with *B. graminis* f. sp. *tritici*, AvrPmaA1 and CSEP0064 were found to enhance local susceptibility (Figure 4.8.a). The enhancement of susceptibility was restricted locally with no effects on second leaves.

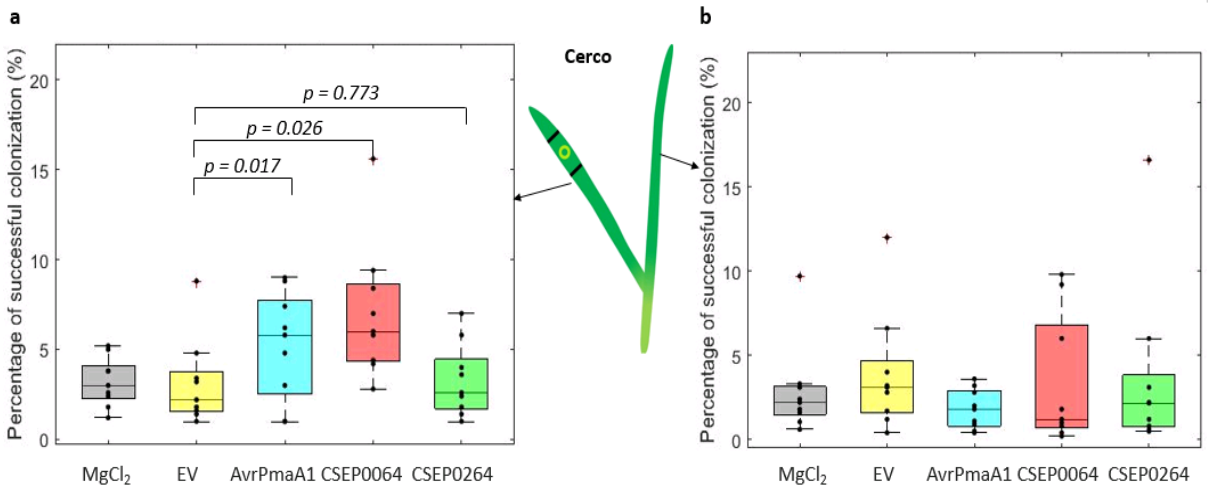

Figure 4.8 Effects of EtHAn-infiltration on local/systemic resistance to *B. graminis* f. sp. *tritici* in Cerco. (a) Compared to EV, spores were more likely to develop colonies on AvrPmaA1 and CSEP0064-infiltrated first leaves. The differences between them and EV were significant, indicated by *p* values both smaller than 0.05. (b) There was no obvious difference of the frequencies of colony formation between different treatments. Data collected from n = 27 samples.

Hardi was a line from the WAGTAIL mapping population that gave no visible responses to CSEPs during screening. Unlike other four lines tested, EtHAn-infiltration did not influence the disease resistance to *B. graminis* f. sp. *tritici* in Hardi significantly, neither systemically nor locally. Although EtHAn-infiltration with effectors seemed to inhibit fungal colonization on the first leaves, the differences were not statistically significant (Figure 4.9.a).

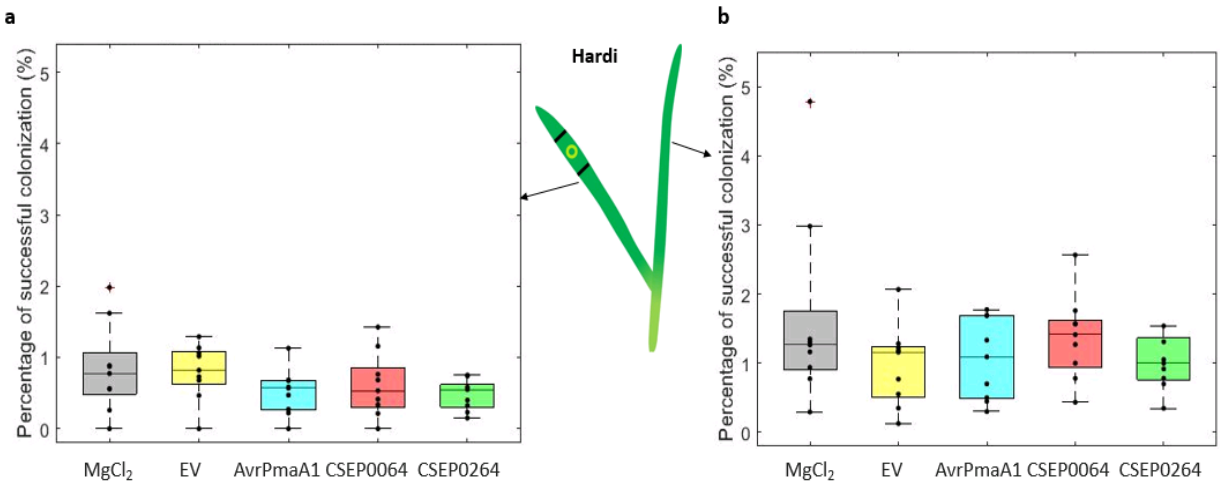

Figure 4.9 EtHAn-infiltration did not influence the disease resistance to *B. graminis* f. sp. *tritici* in Hardi. Boxes plotted for n = 12 samples. No significant difference was observed in the percentages of colony formation between different treatments on first leaves (a) or second leaves (b).

4.5.3 Effects of EtHAn-infiltration on wheat immunity to B. graminis *f. sp. hordei*

I next investigated if EtHAn-infiltration with effectors can affect the susceptibility of wheat to non-adapted pathogen *B. graminis* f. sp. *hordei*. As described above, AvrPmaA1 and two CSEPs triggered systemic resistance to *B. graminis* f. sp. *tritici* infection in Rialto. After counting the number of colonies on *B. graminis* f. sp. *hordei* inoculated plants, I observed reduced susceptibility on the second leaves of effector-infiltrated Rialto (Figure 4.10). However, the differences between them and EV were not statistically significant.

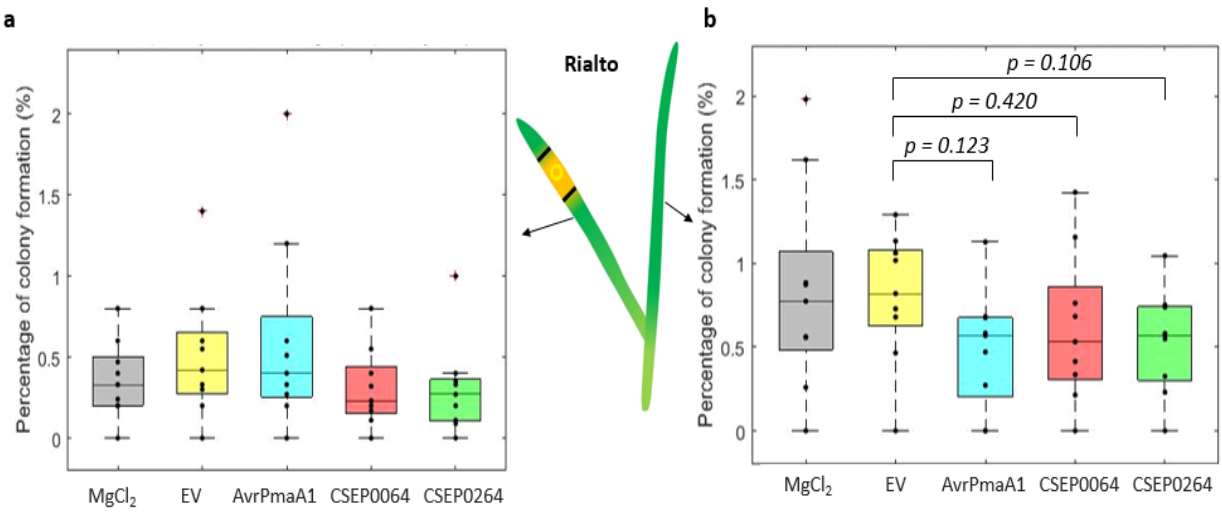

Figure 4.10 *B. graminis* f. sp. *hordei* development on EtHAn-infiltrated Rialto plants. Box plots for n = 27 samples with whiskers extending to a maximum of 1.5 x IQR. Each single dot shown was the mean of three biological replicates. Significant difference between different effectors and EV was defined by Student's *t*–test. (a) Fungal development on first leaves. Very few spores developed colonies in all five tested events. (b) Reduction in colony formation percentage showed in plants that infiltrated with avirulence protein, CSEP0064 or CSEP0264. The differences between them and EV were not significant with *p* values of 0.123, 0.420 and 0.106, respectively.

Unlike the triggering of systemic resistance in Rialto, EtHAn-infiltration with effectors induced systemic susceptibility in Abbot when challenged by *B. graminis* f. sp. *tritici*. As shown in Figure 4.11, the effectors did not compromise the resistance to *B. graminis* f. sp. *hordei* systemically. Conversely, a significant decrease of colony formation frequency was observed in AvrPmaA1-infiltrated plants, suggesting that the bacterial avirulence effector triggered the systemic resistance to non-adapted *B. graminis* f. sp. *hordei*. CSEP0064 and CSEP0264 showed non-significant effects on susceptibility, in this wheat variety.

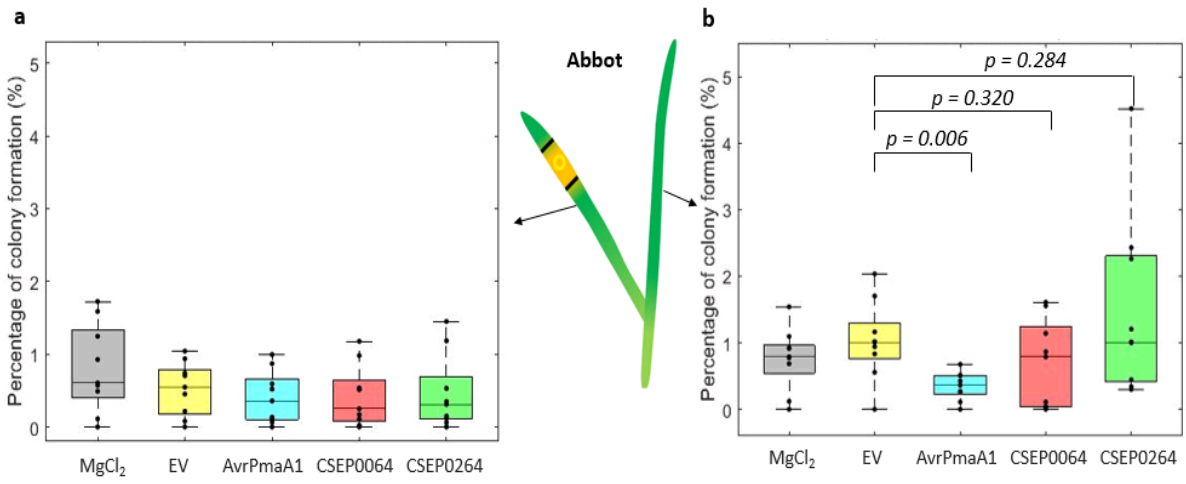

Figure 4.11 Effects of EtHAn-infiltration on local/systemic disease resistance to *B. graminis* f. sp. *hordei* in Abbot. (a) Frequency of colony formation on first leaves. No significant difference was observed between all five treatments. (b) Fungal development on second leaves. AvrPmaA1 significantly (p = 0.006) inhibited the formation of colonies, whereas CSEP0064 or CSEP0264 did not. Data collected from n = 24 samples.

Following this, I tested whether Madrigal, Cerco and Hardi had altered susceptibility to the non-adapted mildew after EtHAn-infiltration. CSEP0264 induced a slight, but not significant, decrease in susceptibility on second leaves of Madrigal (Figure 4.12). Five treatments conferred similar levels of susceptibility to *B. graminis* f. sp. *hordei* in Cerco (Figure 4.13) and Hardi, both locally and systemically.

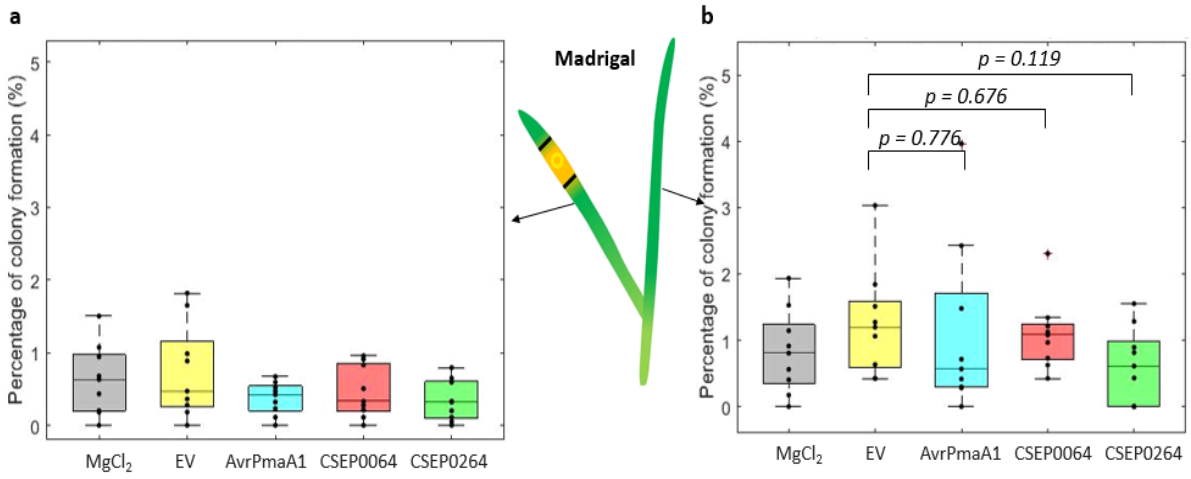

Figure 4.12 Effects of EtHAn-infiltration on local/systemic susceptibility to *B. graminis* f. sp. *hordei* in Madrigal. Boxes plotted for n = 12 samples. (a) Five treatments allowed similar proportions of spores to develop colonies on first leaves. (b) CSEP0264 did not significantly enhance the systemic resistance to *B. graminis* f. sp. *hordei*.

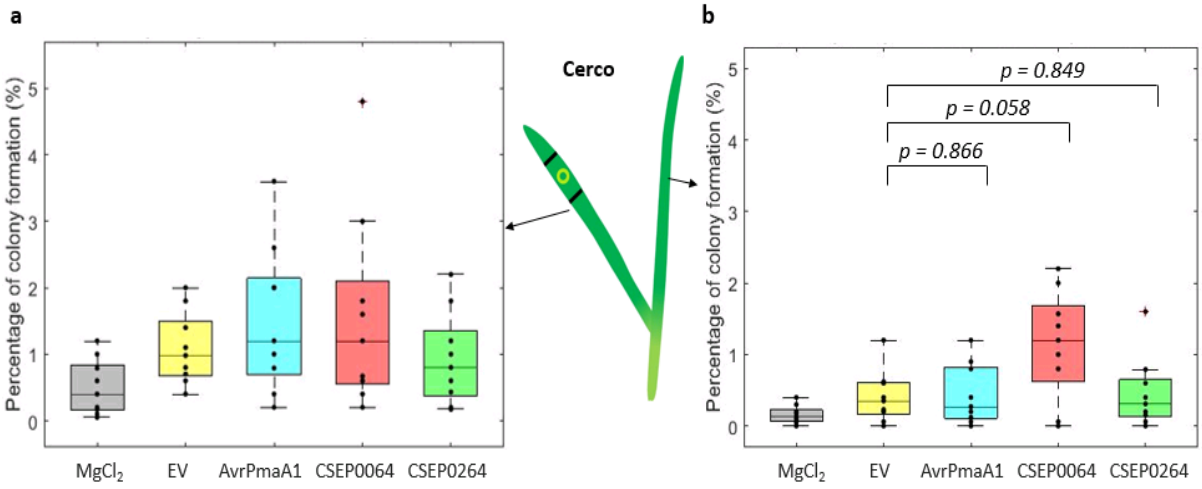

Figure 4.13 Effects of EtHAn-infiltration on local/systemic disease resistance to *B. graminis* f. sp. *hordei* in Cerco. (a) Frequency of colony formation on first leaves. No significant difference was observed between all five treatments. (b) Fungal development on second leaves. CSEP0064 slightly increased the percentage of colony formation, but without significance. n = 27 samples.

4.6 Discussion

In this study, I investigated the effects of EtHAn-delivered effectors wheat immune responses, locally and systemically. Infiltrated plants were challenged by the adapted pathogen *B. graminis* f. sp. *tritici* or non-adapted pathogen *B. graminis* f. sp. *hordei*. A summary of the results is given in Table 4.2. Successful penetration, which is indicated by haustorial development and colony formation, has been used to determine the susceptibility of plants to powdery mildews in various studies. For example, *Arabidopsis* to *B. graminis* f. sp. *hordei* (Collins et al., 2003), barley to *B. graminis* f. sp. *hordei* (Scheler et al., 2016) and wheat to *B. graminis* f. sp. *tritici* (Tang et al., 2017). I therefore chose to observe the colony formation on inoculated plants to investigate the susceptibility change in wheat. Although wounding caused by needles has been shown to alter plant immune responses (Heil et al., 2012, Garcia et al., 2015), wounding by infiltration ($MgCl_2$ mock infiltration) had no effect on wheat immunity change. Leaf infiltration with soil bacteria has been reported to act as PAMPs to trigger SAR in *Arabidopsis* (Niu et al., 2016). However, in this study, the degree of penetration success was similar in EV and $MgCl_2$ consistently in all events, meaning that local PTI triggered by EtHAn-EV did not affect wheat susceptibility to later fungal infections.

One important feature of this study is that I investigated the induced local and systemic immunity in wheat plants simultaneously. Resistance against *B. graminis* f. sp. *tritici* on untreated distant leaves was altered in Rialto, Abbot or Madrigal, whereas local induction of resistance was not observed. Conversely, EtHAn-delivered effectors promoted fungal colonization locally in Cerco, while showed no effect systemically. It has been reported previously that different induced responses can occur in local and systemic tissues (Jordá and Vera, 2000, Savatin et al., 2014). For example, wounding in pepper induces local resistance but systemic susceptibility to later *Botrytis cinerea* infection (Garcia et al., 2015). The accumulation and expression of some defense-related factors, such as hydrogen peroxide,

jasmonate and PR genes have been compared between local and systemic sites (Jordá and Vera, 2000, Kombrink and Schmelzer, 2001). Compared to published results in other systems, my results showed both similarities and differences. As summarized in Table 4.2, CSEP0264 did not induce a higher level of chlorosis than EV in infiltrated local leaves but triggered systemic susceptibility in Abbot, while in Madrigal CSEP0264 enhanced EV-triggered PTI and showed no effect on inducing systemic resistance. The discrepancy suggested that the systemic susceptibility change I observed did not correlate with local defense responses (chlorosis/necrosis). A similar example is that Agroinfiltration of *Nicotiana* with the potato *Rx* gene triggers SAR in the absence of local HR (Liu et al., 2010b).

Table 4.2 Summary of the relationships between systemic immunity and effectors-triggered local responses (chlorosis) in wheat. Effectors were EtHAn-infiltrated into the first leaves. Wheat lines responding to specific effectors are shown with green background. In comparison with EV control, effectors triggered systemic resistance/susceptibility to *B. graminis* f. sp. *tritici*/*B. graminis* f. sp. *hordei* in the second leaves of some lines. Significant differences are indicated as red asterisks (*$p < 0.05$, **$p < 0.01$, Student's *t* test); ns means that the difference between effector and EV was not significant. Enhanced systemic resistance and susceptibility are indicated as '+' and '-', respectively.

Wheat variety		EtHAn-infiltration with different effector		
	Subsequent infection	AvrPmaA1	CSEP0064	CSEP0264
Rialto	*B. graminis* f. sp. *tritici*	(+)**	(+)**	(+)**
	B. graminis f. sp. *hordei*	ns	ns	ns
Abbot	*B. graminis* f. sp. *tritici*	(–)*	(–)**	(–)*
	B. graminis f. sp. *hordei*	(+)**	ns	ns
Madrigal	*B. graminis* f. sp. *tritici*	(+)*	(+)*	ns
	B. graminis f. sp. *hordei*	ns	ns	ns
Cerco	*B. graminis* f. sp. *tritici*	ns	ns	ns
	B. graminis f. sp. *hordei*	ns	ns	ns
Hardi	*B. graminis* f. sp. *tritici*	ns	ns	ns
	B. graminis f. sp. *hordei*	ns	ns	ns

The most critical observation of this study is that EtHAn-infiltration with effectors triggered systemic resistance or susceptibility to subsequent challenge with *B. graminis* f. sp. *tritici* in wheat. In the context of dicots, much of the knowledge of effector or ETI-triggered SAR have been obtained from the interactions between phytopathogenic bacteria and host plants. For instance, *P. syringae* effectors AvrPpt2 and AvrRpm1 elicit SAR in *Arabidopsis* (Alberto et al., 2010, Andersson et al., 2006). To understand the functions of other pathogen (non-bacterial) effectors during induced defense responses, T3SS has been used. Downy mildew effectors fused to *P. syringae* AvrPps4 are delivered by *P. syringae* pv. *tomato* and promote disease susceptibility in *Arabidopsis* (Sohn et al., 2007). Oomycete effectors fused to *P. syringae* AvrRpm1 trigger resistance against later bacterial and oomycete infections in *Arabidopsis* (Rentel et al., 2008). However, the activation of resistance or susceptibility described in those papers is restricted to local responses. SAR or SAR-like phenomena have been reported in monocots in recent years (Balmer et al., 2013b, Dey et al., 2014). Nevertheless, none of them demonstrated the induction of systemic resistance by effectors. Here, CSEPs, which are effectors of the non-host pathogen *B. graminis* f. sp. *hordei*, were delivered into wheat by bacteria through a modified T3SS and resulted in facilitation or inhibition of fungal colonization on the second leaves. My results, which focused on leaf-to-leaf responses, revealed an effector-mediated SAR-like or SAS-like state in wheat for the first time.

An interesting question that arises from this study is why the same set of effectors (AvrPmaA1, CSEP0064 and CSEP0264) triggered systemic resistance in Rialto, while induced systemic susceptibility in Abbot? The genotype of plants was suggested to play a pivotal role in induced defense (Bruce, 2014). Six different grapevine cultivars were pre-treated with BTH, and then varying degrees of efficacy in reducing downy mildew symptoms were observed (Banani et al., 2014). Likewise, six tomato accessions were shown to differ significantly in the inducibility of resistance against *P. infestans* one week after treatment with chemical inducers

(Sharma et al., 2010). The genotype-mediated variation in induced responsiveness has also been reported in monocots. For example, barley plants were inoculated with chemical elicitors, and the expression of induced resistance against *Rhynchosporium secalis* is influenced by the host genotype (Walters et al., 2011). Apart from host genotype, the induction of disease responses can depend on several other factors, such as the organs involved (Blodgett et al., 2007) and the environment (Durrant and Dong, 2004). Given that all immunity tests were performed under the same conditions, I propose the differences in induced systemic responses found here are due to the genotype difference between Rialto and Abbot. A similar result has been found in the peach-aphid system. Preinfestation with *M. persicae* initiates the activation of resistance and susceptibility to secondary infestation among five different genotypes of peach (Sauge et al., 2006). In that case, the induction of resistance and susceptibility is at a local level on a terminal growing shoot. As far as I am aware, there is no published research describing the effects of host genotype on induction of systemic resistance and susceptibility.

In comparison to the effector-mediated alteration in systemic immunity against *B. graminis* f. sp. *tritici*, there was no significant effects caused by effectors on the systemic resistance to *B. graminis* f. sp. *hordei*. The only exception was that AvrPmaA1 induced systemic resistance to *B. graminis* f. sp. *hordei* in Abbot (where systemic susceptibility was enhanced against *B. graminis* f. sp. *tritici*). These findings might suggest that wheat non-host resistance and host resistance are controlled by different systemic signalling pathways: the signalling of SAR-like responses CSEPs triggered may be essential for or shared by host resistance, but inefficient for or different from non-host resistance. However, as the plants had been infiltrated with *B. graminis* f. sp. *hordei* effectors and displayed local defense responses, additional defense activation would not lead to detectable changes (systemic resistance or susceptibility) in the case of non-adapted pathogen. Therefore, another alternative explanation would be that basal

plant immunity is sufficient to restrict non-adapted pathogen invasion and additional up-regulation of systemic immunity does not provide any further advantage.

The SAR signalling has not been clearly elucidated in wheat, but its complexity has been also suggested by various studies. For example, *PR1* genes are induced upon infection with *B. graminis* f. sp. *tritici* but do not respond to SAR inducers SA, BTH or INA (Molina et al., 1999). Similarly, Bertini *et al.* (2013) observed no expression change of *PR1* or *PR5* upon treatments with SA, BTH and MeJA. But, *PR4* genes are upregulated after SAR inducer treatment, as well as wounding in young shoots and leaves (Bertini et al., 2003). However, a more recent study demonstrated the contribution of G3P in triggering SAR in wheat against a rust fungus, and the *TaGLI1*-mediated synthesis of G3P is associated with SA accumulation and *PR1* gene expression (Yang et al., 2013). Intriguingly, *TaGLI1* is induced by *B. graminis* f. sp. *tritici* infection (Li et al., 2016). The same study also found that application of glycerol induces wheat resistance to *B. graminis* f. sp. *tritici*, which is associated with induction of G3P, *PR* genes (*PR1, PR2, PR3, PR4* and *PR5*) and SA. Taken together, two studies about G3P suggested the signalling overlaps between wheat-*B. graminis* f. sp. *tritici* interactions and SAR-like responses.

AvrPmaA1, CSEP0064 and CSEP0264 affected systemic immunity in Rialto and Abbot. In contrast, AvrPmaA1 and CSEP0064 were able to induce SAR-like responses in Madrigal, whereas CSEP0264 did not. What makes CSEP0264 behave differently from the others? This could be simply due to the absence of the host targets of CSEP0264 in inducing systemic resistance. Nevertheless, fewer samples (n=12 rather than e.g., 27 for Rialto) were investigated for Madrigal, due to time constraints. To further confirm the systemic responses triggered by CSEPs in Madrigal and make it comparable with other lines, more replicates of Madrigal are needed.

This study raises new challenges in identification and detection of known SAR signals and markers which would help us to broaden our understanding of SAR in wheat. For example, expression profiling of *PR* genes, measurement of SA, JA and G3P in both local and distant leaves and so on. It may also be interesting to investigate whether the observed induced resistance affects other adapted pathogens as SAR is considered to be broad-spectrum.

4.7 Conclusions

This study showed that recognition of EtHAn-delivered effectors can trigger the systemic enhancement of resistance or susceptibility against *B. graminis* f. sp. *tritici* in wheat. The induction of SAR-like and SAS-like responses is influenced by host genotype. Compared to the effects of CSEPs on elicitation of systemic responses against host pathogen *B. graminis* f. sp. *tritici*, delivered-CSEPs do not affect systemic wheat immunity to subsequent infection of the non-adapted pathogen *B. graminis* f. sp. *hordei.* Moreover, the systemic immunity change does not correlate with local chlorotic/necrotic responses.

5 Detection of CSEP0064 in infected barley leaves

5.1 Introduction

Understanding the role and function of effector proteins requires the knowledge of their localization and host targets (Varden et al., 2017). Computational and mass spectrometry-based approaches enable the investigations of the existence of CSEPs in different structures (Bindschedler et al., 2009, Pliego et al., 2013). Immunoprecipitation coupled with MS and yeast-2-hybrid (Y2H) have been used to identify the host targets of CSEPs (Ahmed et al., 2016, Pennington et al., 2016). In addition to these, profiling of protein expression also contributes to uncovering the biological activities of CSEPs. To date, quantitative real-time PCR (qPCR) has been used to determine the expression patterns of CSEPs (or BECs) in different studies. Using qPCR, eight CSEPs including CSEP0064 were found up-regulated in the early stages of infection and formation of haustoria (Pliego et al., 2013), silencing of BEC1019 (confirmed by qPCR) was found to reduce fungal colonization (Whigham et al., 2015), 22 CSEPs were suggested to be involved in initial fungal aggressiveness (Ahmed et al., 2015), CSEP0081 and CSEP0254 were shown to dramatically increase their transcript abundances during the establishment of haustoria (Ahmed et al., 2016) and five CSEPs from the same family to which CSEP0064 belongs were noticed to express non-simultaneously during fungal development (Pennington et al., 2015). Although transcript abundance can be measured by qPCR, it is generally accepted that mRNA transcript levels may not always correlate with protein levels (Maier et al., 2009, Hurt et al., 2014, Mahmood and Yang, 2012). Beyond transcriptional level, the expression level of a protein is determined by many other factors, for example, post-transcriptional regulation, translation rate, protein stability and protein modification (Liu et al., 2016b). Therefore, monitoring synthesized mature/active protein in tissues also plays a pivotal role in understanding protein activity and function.

For decades, western blotting has been routinely used in various fields for different purposes, for example, measuring protein abundance, determining cellular localization and monitoring post-translational modifications (Mahmood and Yang, 2012, Bass et al., 2017). In a western blotting system, proteins are separated by molecular mass, the existence of a unique protein is detected by highly specific antibody and the abundance of protein present is determined by the thickness/intensity of the band (Gallagher et al., 2008). Western blot is also extensively used in researches of plant-microbe interactions including but not limited to barley and *B. graminis* f. sp. *hordei* (Lu et al., 2016). For instance, a *B. graminis* f. sp. *hordei* peptide was found to interact with barley GTPase HvRACB, to contribute to virulence and this peptide is detectable in infected barley leaves by western blot (Nottensteiner et al., 2018). Surprisingly, few studies have been done to directly characterize CSEPs in powdery mildew-infected host tissues using western blot. The anti-CSEP0064 polyclonal primary antibody (raised in rabbits) provided the possibility of determining the expression of this effector protein in host plant based on its protein level rather than mRNA level. In addition to that, any post-translational modifications or protein-protein interactions of CSEP0064 would be identified through western blot analysis.

Post-translational modification (PTM) enables rapid and reversible alterations of proteins by covalent or enzymatic modification. PTM has proven to play diverse roles in host-pathogen interactions (Takahashi et al., 2009, Walley et al., 2018). In numerous studies of medical important fungi, such as *Saccharomyces cerevisiae, Candida albicans* and *Aspergillus fumigatus*, PTMs were found to contribute to pathogenicity through driving structural contributions to target proteins, determining the stability and localization of target proteins and many other ways (Leach and Brown, 2012, Beltrao et al., 2009, Lopes da Rosa et al., 2010). These modifications include ubiquitination, glycosylation, phosphorylation, acetylation, small ubiquitin-like modifier (SUMO)-ylation and so on (Miura and Hasegawa, 2010).

In the context of plant-microbe interactions, PTMs play vital roles for both plant and pathogen in this complex system of molecule and signaling exchanges. In plant cells, PTMs are employed to control and target efficiently the activity of innate immune regulators to accelerate the defense responses (Bhattacharjee et al., 2015, Withers and Dong, 2017). As an example, a SUMO ligase E3 increases the resistance of *Arabidopsis* to *P. syringae* pv. *tomato* DC3000 by increasing the expression levels of *PR* (pathogenesis-related) genes (Lee et al., 2007). In another study of *Arabidopsis*, phosphorylation of receptor-like kinases was found to initiate plant immune responses to *P. syringae* pv. *tomato* DC3000 (Lin et al., 2014). While plants utilize PTMs to regulate their immune system, pathogens also use PTMs to suppress plant immunity by manipulating plant proteins directly or indirectly (Salomon and Orth, 2013, Howden and Huitema, 2012). For example, two type III effectors AvrB and AvrRpm1 of *P. syringae* phosphorylate a negative regular of basal defense in *Arabidopsis*, leading to activation of an immune receptor *RPM1 t*o mount immune responses (Chung et al., 2011). Similar results of effector-mediated PTMs triggering host immune systems have been found between *P. syringae* pv. *tomato* DC3000 and tomato, *X. campestris* and *Arabidopsis* as well (Feng et al., 2012, Rosebrock et al., 2007).

Recently, PTMs such as *N*-glycosylation of plant-pathogenic fungal proteins has been investigated but is still poorly understood (Salomon and Orth, 2013). *N*-linked glycosylation is one of the most ubiquitous PTMs. The glycan moiety is synthesized in endoplasmic reticulum (ER) and subsequently processed in the Golgi apparatus, where it plays a role in protein sorting, transport, folding, quality control and recognition (Helenius and Aebi, 2004, Motteram et al., 2011) . *N*-glycosylation is suggested to be a common feature of fungal effectors due to the identification of increasing number of *N*-glycosylated effectors in different fungi (Chen et al., 2014a, Fang et al., 2016). More importantly, *N*-glycosylation of effectors was found to contribute to fungal pathogenesis through different mechanisms (Fernandez-Alvarez et al.,

2013, Motteram et al., 2011, Schirawski et al., 2005). For instance, attachment of three *N*-glycans onto the Secreted LysM Protein1 (Slp1) is required for rice blast fungus *M. oryzae* to evade rice innate immunity (Chen et al., 2014a), and two *N*-glycosylated RNase-like effectors of rice false smut fungus were identified to induce cell death or trigger defense responses in rice and *N. benthamiana* (Fang et al., 2016). In comparison to *N*-glycosylation, other types of glycosylation like *O*-glycosylation have received little attention in phytopathogenic fungi.

As mentioned previously in Chapter 1, two RNase-like effectors CSEP0064 and its paralog CSEP0264 were considered to be secreted by the powdery mildew haustoria (Pliego et al., 2013). But the mechanisms behind powdery mildew effector delivery/release into host cytosol are still unclear. Sec61 is known as an ER membrane protein translocator that consists of Sec61α, Sec61β and Sec61γ. In barley, Sec61β is considered to be an essential component for retrotranslocon activity and silencing of it can reduce the susceptibility of barley to *B. graminis* f. sp. *hordei* (Zhang et al., 2013, Kelkar and Dobberstein, 2009). Zhang et al. (2013) also found that GFP-labelled barley Sec61β localizes proximally to the haustorial body. They further proposed a Sec61-dependent model and suggested that *B. graminis* f. sp. *hordei* effectors travel to host cytosol through the Sec61 retrotranslocon pores in ER with or without passing Golgi. Therefore, I hypothesize that effectors can be modified during the release process. In this chapter, I will use western blot analysis of CSEP0064 in infected barley tissues to test this hypothesis.

5.2 Objectives

1. To utilize western blot to investigate the expression level of CSEP0064 in infected whole barley leaves and dissected tissues.

2. To identify and validate the possible modification of CSEP0064.

5.3 Materials and Methods

5.3.1 Plant materials

Barley (*H. vulgare*) cv. Golden Promise seedlings and *B. graminis* f. sp. *hordei* were maintained as described in 4.3.1. The first leaves of barley seedlings were collected from 0 (non-inoculated) up to 9 days post inoculation (dpi) (when they are heavily infected).

5.3.2 Protein sample preparation

Ten barley leaves were ground to fine powder with liquid nitrogen. The powder was then processed using different extraction methods/buffers described below.

5.3.2.1 Native extraction

The powder was transferred into a 1.5 ml Eppendorf tube and lysed in 500 µl of extraction buffer (0.01% Tween-20, 50mM sodium phosphate buffer pH 8, 300mM NaCl, 1mM $MgCl_2$, 1% PVPP). Protease inhibitor (ProteoGuard EDTA-free protease inhibitor cocktail, Takara Bio, USA) was added just before use to a final concentration of 0.1% (v/v). The mixture was incubated on ice for 10 min, followed by 20-min centrifugation at 13,000 *g* on a table-top Eppendorf microcentrifuge at 4 ℃. Then the supernatant was carefully transferred to a new 1.5 ml tube.

5.3.2.2 TCA/Acetone protein precipitation

To precipitate proteins, 4 ml of cold TCA solution (10% (w/v) TCA and 0.07% (v/v) β-mercaptoethanol in acetone) was added to the powder in a 15 mL tube. The mixture was then incubated at -20 ℃ for 1 h. Insoluble particulates were then separated from the soluble fraction by centrifugation at 13,000 *g* for 20 min at 4 ℃. To remove excess TCA, the protein pellet was washed five times with cold acetone (with 0.07% β-ME) till the supernatant is not acid (tested using pH indicator strips). After a last wash, all liquid was removed and the pellet was allowed to stand on the bench for at least 2 min to let the acetone evaporate. Pellet was then resuspended

in denaturing extraction buffer (7M Urea, 2M Thiourea, 10mM DTT, 0.1% CHAPs and 0.1% (v/v) protease inhibitor) and incubated at room temperature for 30 min. Finally, the sample was centrifuged for 20 min at 13,000 *g* to separate the supernatant.

5.3.2.3 Nuclease treatment

Proteins extracted with native lysis buffer were treated with DNase I (Cat.2222, ThermoFisher Scientific) or RNase A (EN0531, ThermoFisher Scientific) according to the manufacturers' instructions for 15-30 min on ice when needed. Benzonase Nuclease (E1014, Sigma-Aldrich) was added to the sample, and the incubation lasted for 30-60 min at 37 ℃.

5.3.2.4 Deglycosylation by enzyme

To remove *N*-linked oligosaccharides by enzyme, proteins were treated with PNGase F (P7367, Sigma-Aldrich). First, 12 µl proteins (total 50 µg) were denatured at 95 ℃ for 5 min with 0.5% SDS and 0.1 M DTT. Once completed, the sample was cooled to room temperature, and mixed with 2 µl TBST to reach pH 6-10. After adding 2 µl of 10% Triton X-100, the sample was then incubated with 2 µl of recombinant PNGase F for 2 h at 37 ℃. Glycoprotein RNase B was included as a positive control to show the efficiency of PNGase F.

5.3.2.5 Deglycosylation by chemicals

Proteins were prepared using the TCA/Acetone precipitation method and dried under nitrogen thoroughly. TFMS was mixed with anisole (10% (v/v) final concentration) and cooled to 0 ℃ before adding to protein sample. Subsequently, the reaction mixture was incubated on ice for 1 h with occasional shaking. After incubation, the mixture was placed in dry ice for several minutes, and then neutralized with 0.2 M Tris solution (same volume of TFMS used). The deglycosylated protein contained in the neutralized reaction mixture was finally precipitated by ethanol (see 5.3.2.3 for protocol).

Proteins were prepared by TCA/Acetone precipitation and thoroughly dried under a stream of nitrogen. One hundred microliters of 5 mg/ml Borane-ammonia (Sigma-Aldrich, Germany) was added to the sample and incubated at 45 ℃ overnight. Excess ammonia was removed by flushing the sample with nitrogen.

Sodium hydroxide was added to proteins extracted with denaturing buffer to a final concentration of 0.1 M and incubated at 50 ℃ for 8 hours.

5.3.2.6 Other chemical treatments

TCEP (Tris (2-carboxyethyl) phosphine) was used to reduce disulfide bonds in proteins. Proteins extracted with native extraction buffer were incubated with 10 mM TCEP overnight at room temperature, finalised by adding 0.2 M Tris solution drop by drop till pH ≈ 7 (tested by pH indicator strips).

Pellets containing proteins precipitated with TCA/Acetone were dissolved in 200 µl of trifluoroacetic acid (TFA) and vortexed thoroughly. The mixture was centrifuged at 13,000 *g* on a table-top centrifuge for 20 min at room temperature, and the tube containing the pellet was placed in a fume hood under a stream of nitrogen to let the TFA evaporate for 1 h. The residue was dissolved in denaturing extraction buffer for protein extraction (see details above in 5.3.2.2).

5.3.2.7 Immunoprecipitation

Ground leaf powder was lysed with immunoprecipitation lysis buffer (10% glycerol, 25 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl, 2% (w/v) PVPP, 10 mM DTT, 0.1% Tween-20 and 0.1% (v/v) protease inhibitor) on ice. After 20 min, the lysates were centrifuged at 13,000 *g* for 20 min at 4 ℃ to remove the cell debris. Then the samples were mixed with more IP lysis buffer to a final concentration of 5 mg/ml. An amount (100-150 µl) of magnetic SureBeads (Bio-Rad, USA) was transferred to a 1.5 ml Eppendorf tube and prewashed for three times with 1 ml pre-

cooled PBST (3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, 135 mM NaCl and 0.05% Tween-20, pH 7.4). Ten µg of antibody (anti-CSEP0064, raised in rabbits) in 200 µl PBST was added to the beads, and then rotated at RT for 10 min. The beads coated with antibody were then isolated using a magnet stand and washed three times, followed by incubating with 100 µg of total protein lysate at RT for 1 h on a rotation wheel. After washing for three times, the bound complexes were eluted by boiling the beads in sample buffer (see 5.3.3.1) for 5 min at 100 ℃.

Anti-CSEP0064 primary antibody was purified using a method based on immunoprecipitation. After incubating the original antibody with magnetic SureBeads, 1 ng of recombinant CSEP0064 was added to the beads instead of total protein lysate. The beads were thoroughly washed after one hour's rotation. The bound complexes were then eluted with 40 µl of 20 mM Glycine (pH 2.0) and neutralized with 0.2 M Tris solution. Finally, the mixture was used as primary antibody in western blot analysis.

Protein concentration of all samples was estimated by Bradford assay (Bio-Rad, USA).

5.3.3 SDS-PAGE

5.3.3.1 Tris-Glycine system

The resolving buffer was made with 1.5 M Tris-hydrochloride (pH 8.8), and the stacking buffer was made with 1.0 M Tris-hydrochloride (pH 6.8). The amounts of reagents required for stacking and separating gels are given in the table below.

Table 5.1 Recipes for casting Tris-Glycine mini gels. A/B for 30% acrylamide/ bisacrylamide (37.5:1) solution (Sigma-Aldrich, Germany), SDS for sodium dodecyl sulphate and APS for ammonium persulphate.

		Stacking gel	Resolving gel		
		5%	10%	12%	15%
Water	(ml)	6.8	4.0	3.3	2.3
A/B	(ml)	1.7	3.3	4.0	5.0
1.5 M Tris (pH 8.8)	(ml)	X	2.5	2.5	2.5
1.0 M Tris (pH 6.8)	(ml)	1.25	X	X	X
10% (w/v) SDS	(µl)	100	100	100	100
10% (w/v) APS	(µl)	100	100	100	100
TEMED	(µl)	10	4	4	4

After the gels polymerized, protein samples were mixed with 4x sample buffer (200mM Tris-Cl pH 6.8, 400mM DTT, 8% SDS, 0.04% bromphenol blue and 40% glycerol) and heated at 100 ℃ for 5 min before loading to the gel. Protein ladders (PageRuler^{TM} Prestained and Spectra^{TM} Low Range, ThermoFisher Scientific, USA) were used. The electrophoresis was carried out with 1x Tris-Glycine electrophoresis buffer (25mM Tris, 250 mM Glycine and 0.1% (w/v) SDS) at 100 V for 2 h.

5.3.3.2 Tris-Tricine system

To cast the gels for Tris-Tricine system, Tris-Cl/SDS buffer (3 M Tris-hydrochloride pH 8.45 and 0.3% SDS) was needed. See gel recipes below.

Table 5.2 Recipes for casting Tris-Tricine mini gels. A/B for 30% acrylamide/bisacrylamide (37.5:1) solution.

		Stacking gel	Resolving gel
		4%	10%
Water	(ml)	7.4	3.5
A/B	(ml)	1.6	5.0
Tris-Cl/SDS buffer	(ml)	3.0	5.0
Glycerol	(ml)	X	1.5
10% (w/v) APS	(µl)	90	50
TEMED	(µl)	9	5

Desired amount of protein was heated at 100 ℃ for 5 min after mixed with 4 x sample buffer (0.1 M Tris-Cl/SDS, 48% glycerol, 16% SDS, 0.4 M DTT and 0.04% Coomassie Blue). While waiting, the wells were rinsed with cathode buffer (0.1 M Tris, 0.1 M tricine and 0.1% SDS). Then, the inner chamber was filled with cathode buffer and the anode buffer (0.2 M Tris-Cl, pH 8.9) was added to the outer chamber. The electrophoresis was run at an initial voltage of 30 V for 1 h followed by 150 V for another 1 h. This protocol was adapted from Schagger et al. (2006).

5.3.4 SDS-PAGE gel staining

Proteins separated in the gel can be visualized directly by Coomassie Blue staining, as described by Candiano et al. (2004). The gel was stained overnight with staining solution (10% (v/v) phosphoric acid, 10% (w/v) ammonium sulphate and 0.12% Coomassie Blue G-250) on a rotating platform at 30 rpm. Destaining was carried out with distilled water for several changes till the background was clear.

5.3.5 Western blotting

The proteins were transferred to a PVDF membrane and detected by western blotting as described by Gallagher et al. (2008). A PVDF membrane (Sigma-Aldrich, Germany) was activated with methanol for several minutes before assembling the transfer sandwich in the following order: sponge, filter paper, SDS-PAGE gel, PVDF membrane, filter paper and sponge. The transfer cassette was then immersed with transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol) in a transfer tank (Bio-Rad, USA). The proteins were transferred from gel to membrane for 1 h at 100 V at room temperature.

Once transfer was completed, the PVDF membrane with proteins was taken out of the assembly and blocked with 1x TBST buffer (20 mM Tris-Cl and 150 mM NaCl, pH 7.6, 0.1% Tween-20) with 5 % (w/v) skimmed milk (Marvel) for 1 h on a shaker at room temperature. The membrane was then incubated with 1/10,000 diluted primary antibody (anti-CSEP0064) in 5 % milk with TBST at 4 ℃ overnight. On the next day, the membrane was washed with TBST four times for 10 minutes each, followed by incubation with 1/3000 diluted secondary anti-rabbit IgG HRP (Cat. 170-6515, Bio-Rad) for 1 h at room temperature. Once completed, the membrane was washed for four times as before.

The target proteins were then visualized using the Fujifilm LAS-3000 Luminescent Image Analyzer after incubated with chemiluminescence solutions (Pierce ECL Plus Western Blotting Substrate, ThermoFisher Scientific).

5.3.6 In-gel digestion and MRM-MS

Prior to MRM-MS (discussed in detail in the following chapter), the proteins were separated and stained on SDS-PAGE and then were digested in gel. Protein bands were excised and cut into 1-2 mm^3 blocks, and then transferred to 0.65 ml low binding microcentrifuge tube (Sorenson BioScience, USA). The gel pieces were washed and destained, each time for 10 min,

as following steps: once in 100 µl of 50mM ABC, once in 100 µl of 25 mM ABC, twice in 50 µl of 33% ACN in 25 mM ABC, twice in 40 µl of 50% ACN in 25 mM ABC, followed by 20 min freeze-drying in a freeze dryer (EDWARDS, UK). Dehydrated gel pieces were rehydrated and reduced at 50 ℃ for 45 min in 30 µl of 20 mM DTT in 25mM ABC. Excess liquid was removed and 30 µl of 10mg/ml iodoacetamide in 25 mM ABC was added to alkylate cysteines for 1 h in darkness at room temperature. After three washes with 50% ACN in 25 mM ABC, the gel slices were freeze-dried for 20 min. Rehydrated with 20 µl of 25 mM ABC containing 10 ng/µl trypsin for 10 min, the gel pieces were then incubated with 30 µl of 25 mM ABC overnight at 37 ℃ and stopped by the addition of 30 µl of 50% ACN in 5% TFA. The extraction of peptides was carried out for three times incubation with 50 µl of 50% ACN in 5% TFA and once with 75% ACN in 2.5% TFA. Each pooled extract was freeze-dried and kept at -80 ℃ until MRM-MS.

5.4 Results

5.4.1 Detection of CSEP0064 and discovery of 'Big CSEP'

Western blotting analysis is essential to reveal whether CSEP0064 is detectable in infected barley tissue. Recombinant CSEP0064 was included to show the sensitivity of anti-CSEP0064 antibody. Healthy barley tissue (uninfected, 0 dpi) and anti-CSEP0264 antisera raised in the same species using the same procedures were used as negative controls to determine the specificity of bands detected by anti-CSEP0064 antibody. The expected sizes of recombinant CSEP0064, wild-type CSEP0064 and CSEP0264 are 11.2 kDa, 10.8 kDa and 10.8 kDa, respectively. From the comparison between two antibodies, it was clear that using anti-CSEP0064 I was able to detect at least 1 ng recombinant CSEP0064. Anti-CSEP0264 did not bind to it. A very weak signal at the position on the western blot where the mature CSEP0064 should be was detected in heavily infected barley sample (9 dpi). In addition to this, an

unexpected saturated and clear band showed up at a much higher molecular weight in 9 dpi sample (see Figure 5.1.a below). The signal corresponding to the large entity was barely visible while using anti-CSEP0264 antisera (Figure 5.1.b), suggesting that it may be specific to anti-CSEP0064 antibody. But it may not necessary relevant to CSEP0064. This was further investigated in following experiments.

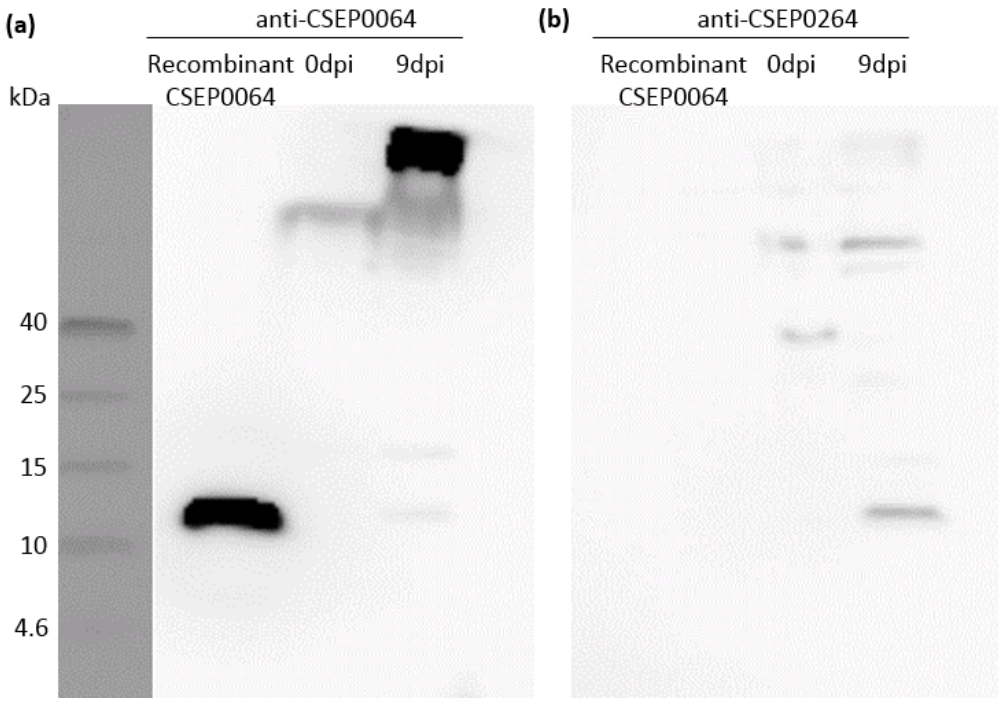

Figure 5.1 Western blot of total barley proteins using anti-CSEP0064 antibody and anti-CSEP0264 antiserum. Low-range protein ladder and two groups of samples: recombinant CSEP0064 (1 ng), uninfected barley (0 dpi) and heavily infected barley (9 dpi) were separated using Tris-Tricine system. (a) One group were subjected to immunoblotting with anti-CSEP0064 primary antibody. (b) The second group was immunoblotted with anti-CSEP0264 primary antiserum.

To investigate at what stage CSEP0064 can be detected, protein extracts across an infection time-course were analyzed by western blot. Barley leaves were sampled from healthy plants (0 dpi) up to 9dpi when the plants were heavily infected by *B. graminis* f. sp. *hordei*. A band at the expected size (10.8 kDa) only appeared in samples from late-stage infection (7 dpi, 8 dpi

and 9 dpi; Figure 5.2, red asterisk), showing that the CSEP0064 accumulated during fungal development and propagation. Similarly, the high molecular weight signal (≥ 200 kDa) which had been observed previously in Figure 5.1 was detected at 3 dpi and saturated at 5 dpi. Therefore, I inferred that this 'intense' signal is relevant to *B. graminis* f. sp. *hordei* infection. Two bands sized just above 15 kDa and 55 kDa (Figure 5.2, black asterisk) existed in all samples, including non-infected plants. These signals are considered to represent non-specific binding of the antibodies with plant proteins.

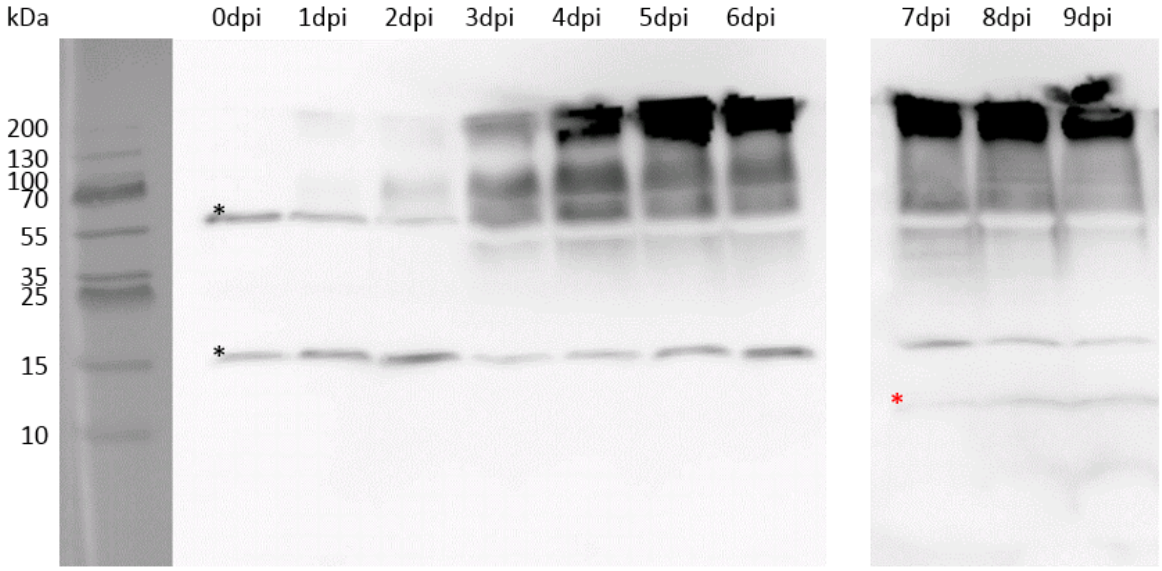

Figure 5.2 Western blot with proteins extracted from barley leaves in a time-course of *B. graminis* f. sp. *hordei* infection. Proteins were extracted by denaturing lysis buffer and separated using Tris-Glycine system together with PageRuler protein ladder, followed by immunoblotting with anti-CSEP0064 primary antibody. Same amount of proteins (25 µg) from different time points were loaded. Two unidentified bands were labeled by black asterisks (*). Target band of CSEP0064 at expected size 10.8 kDa was detectable in 7 dpi, 8 dpi and 9 dpi samples, shown by red asterisk (*).

To optimize the sensitivity of detection of CSEP0064 and the intensity of detected band signal, various factors were investigated including transfer time, SDS-PAGE systems, lysis buffers and quantity of loaded samples. Tris-Tricine system was ideal for separation of small

proteins, for example, wild-type CSEP0064, while Tris-Glycine was needed for better separation of high molecular weight proteins. There was no obvious difference between two lysis buffers for detecting CSEP0064, but denaturing extraction buffer provided higher solubility for big protein complexes (Supplementary Figure 1). A time-course of transfer time was carried out to determine how to achieve the highest transfer efficiency. After western blot analyses of recombinant CSEP0064 and barley leaf total proteins (9 dpi) transferred with 10 min, 20 min, 30 min, 40 min, 50 min and 60 min, respectively, it was found that both bands achieved maximal signal intensity after 60 min transfer (Supplementary Figure 2).

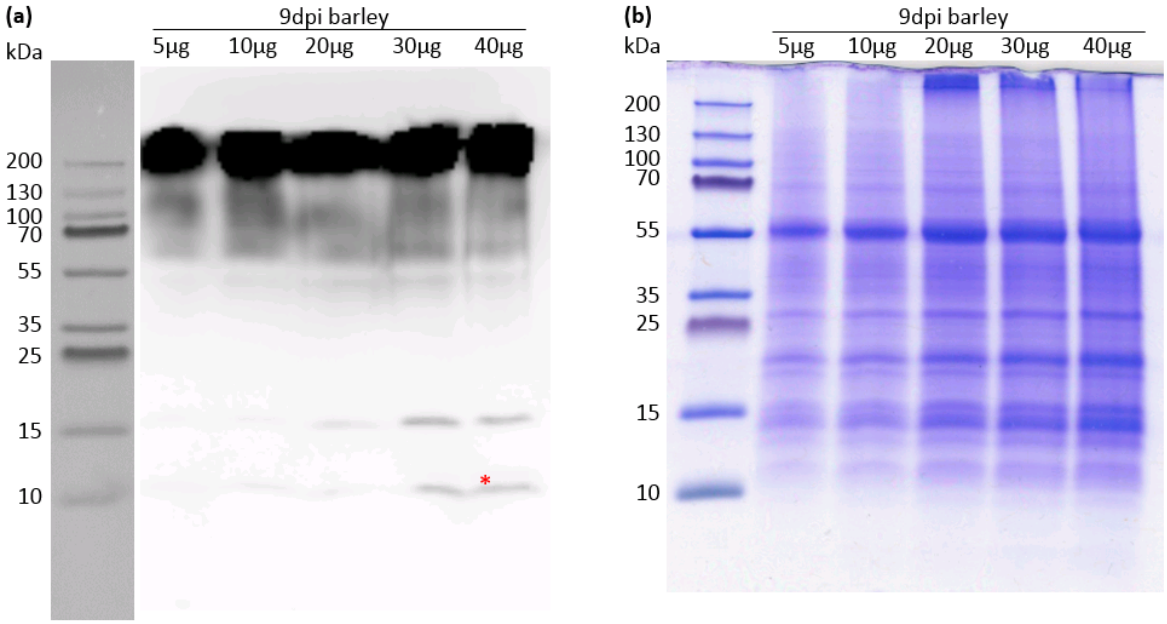

Figure 5.3 Immunoblot analysis of a dilution series of total proteins extracted from infected barley leaves (9 dpi). Proteins were extracted using denaturing lysis buffer and separated by Tris-Tricine system. PageRuler protein ladder was loaded onto the first lane of SDS-PAGE gel. (a) Continued with western blotting. Relatively clear band at expected size of CSEP0064 was detected in 30 µg and 40 µg samples, shown by red asterisk (*). A distinctively strong signal ≥ 200kDa was detected in all samples. (b) Another gel with exactly same samples was electrophoresed, and then stained with Coomassie Blue to show protein loadings.

A dilution series of total proteins from infected barley (9 dpi) was analyzed by western blot to demonstrate at what quantity of loaded protein would give a clear CSEP0064 band. The amount of total proteins loaded were 5 µg, 10 µg, 20 µg, 30 µg and 40 µg. CSEP0064 was detectable when ≥ 30 µg total proteins were loaded, indicating that its expression levels are only just about detectable with this technique in *B. graminis* f. sp. *hordei* infected plants. Conversely, the high molecular weight signal was obviously strong even in 5 µg sample.

As the anti-CSEP0064 antibody I used was a polyclonal antibody, the epitopes it recognized could be shared by many different proteins in addition to CSEP0064, leading to misinterpretation of western blot results. To further investigate whether the high molecular weight signal was due to unspecific binding to anti-CSEP0064 antibody, I attempted to purify it by immunoprecipitation (details in 5.3.2.7). Then a western-blot analysis was performed using the purified antibody (Figure 5.4). A clear band was detected in recombinant CSEP0064 sample, the same as previous results. But in 0 dpi sample, two unspecific bands (at 15 kDa and 55 kDa) that had been observed in Figure 5.2 were no longer detectable by the purified antibody, meaning that the purification of anti-CSEP0064 improved its specificity. Therefore, the presence of an abundant signal around 200 kDa in 9 dpi sample indicated that this signal was CSEP0064-specific. It was then named ‘Big CSEP’ according to its higher molecular weight than wild-type CSEP0064.

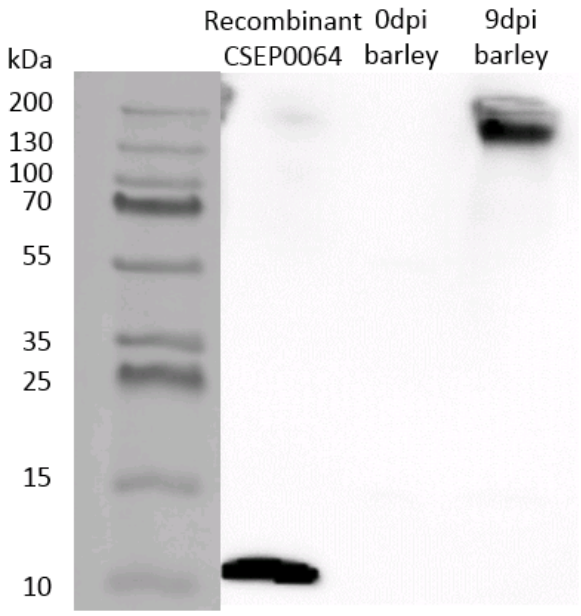

Figure 5.4 Western blotting with purified anti-CSEP0064 primary antibody. Proteins were extracted using denaturing lysis buffer and separated by Tris-Tricine SDS-PAGE gel, and then subjected to western blotting with purified anti-CSEP0064 primary antibody. Recombinant CSEP0064 (1 ng) was used as a positive control and 0 dpi barley (20 µg) as a negative control. Five µg of 9 dpi sample (same with the smallest quantity used in Figure 5.3) was loaded. The high molecular weight band signal was still prominent.

Next, I aimed to investigate where in the infected leaf CSEP0064 and 'Big CSEP' could be detected. Proteins extracted from different tissues were analysed using western blot. Using the methods described above in Chapter 6, infected barley leaves were dissected into epiphytic (EP) layer which contains mycelia on leaf surface, epidermal (ED) layer where haustoria establish, isolated haustoria (HA) and the others left in epidermis after removal of haustoria (ED-HA). As expected, CSEP0064 was detected in HA with a clear band around 11 kDa and the signal of this band was stronger than any band representing CSEP0064 previously detected in whole leaf. Apart from this, the 'Big CSEP' and three other bands sized just above 15 kDa, 25 kDa and 35 kDa were also detected in HA sample (Figure 5.5.a). The 'Big CSEP' appeared in EP and ED as well. The migration of ED-HA and HA proteins on gel showed that they contain a

lower proportion of high molecular weight proteins in comparison with EP and ED (Figure 5.5.b).

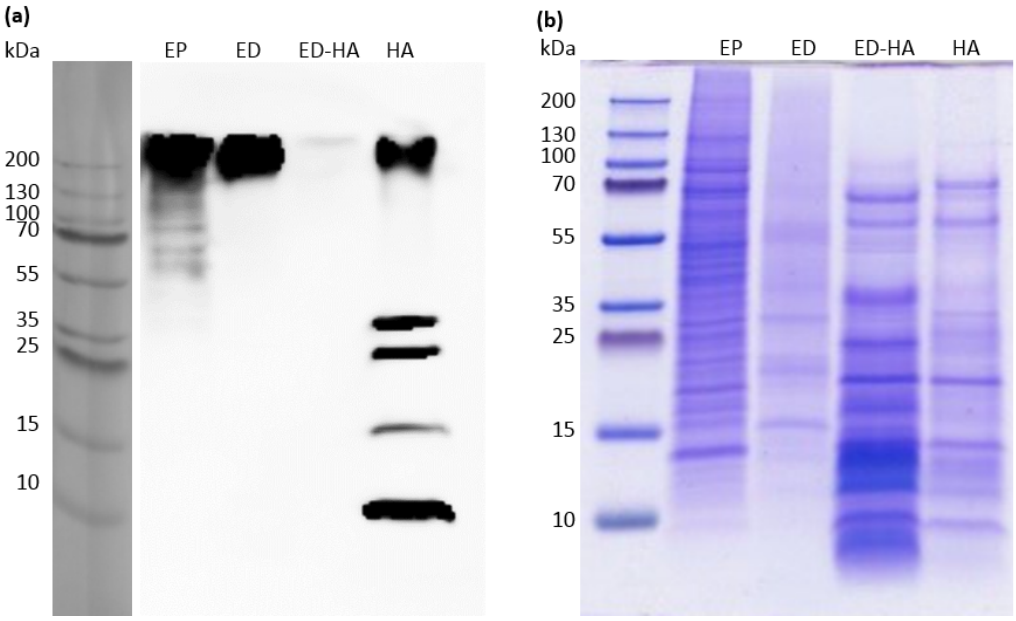

Figure 5.5 Immunoblot analysis of proteins extracted from dissected EP, ED, HA and ED-HA samples with standard anti-CSEP0064 antibody. Same amount of total proteins (20 µg) were loaded and separated on SDS-PAGE gel, then (a) analysed by western blot or (b) visualised by Coomassie blue. The measurement of protein concentrations using Bradford assay may be interfered by chemicals/enzymes used in dissection, so the total protein abundance shown in each sample after staining was more or less different. Strong signal of 'Big CSEP' were detected in EP, ED and HA, whereas CSEP0064 signal at expected size was only detectable in HA.

5.4.2 Disruption of 'Big CSEP' by chemicals and enzymes

To investigate if the 'Big CSEP' is due to protein aggregation, TFA and TCEP were used to dissociate non-covalent interactions and disulfide bonds, respectively (Liu et al., 2010a, Spanu, 1997). TCEP was added to total protein extracts to be a powerful and irreversible reducing agent instead of DTT. Sample treated with TCEP was separated on SDS-PAGE gel and examined by western blot. The result shown in Figure 5.6 (lane 1) indicated that TCEP did not reduce any signal of 'Big CSEP'. Treatment with TFA removed some of the protein bands

(shown in Figure 5.6.b, e.g., the band around 55 kDa) while retaining the 'Big CSEP' signal. It revealed that TFA selectively dissociated proteins with non-covalent bonds.

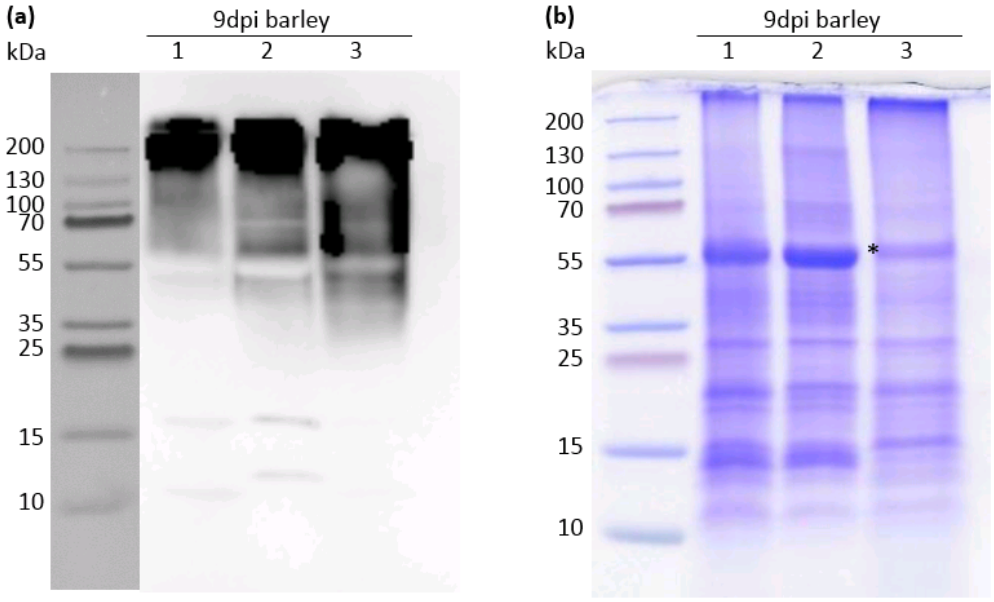

Figure 5.6 Western blot analysis of total proteins (9 dpi) treated with TCEP or TFA. Proteins extracted with native lysis buffer was incubated with TCEP (lane 1). TFA was added to proteins precipitated with TCA/Acetone (lane 3), another sample was treated with water instead of TFA in the exactly same conditions (lane 2). These three samples were separated on a Tris-Glycine SDS-PAGE gel. (a) Western blot. (b) Proteins in the gel stained with Coomassie blue to show the protein loadings. The band around 55 kDa (labeled with an asterisk *) largely reduced in TFA treated sample.

As mentioned in Chapter 1, CSEP0064 is predicted to be an RNase-like protein. I therefore tested whether the 'Big CSEP' could be a complex of CSEP0064 bound with RNA or DNA. To achieve this, Benzonase nuclease was added directly into protein extracts to degrade all forms of DNA and RNA. The result (Figure 5.7.a) clearly showed that the removal of nucleases did not influence 'Big CSEP'.

Subsequently, I tested whether the 'Big CSEP' was a glycosylated protein. Alkaline β-elimination, for example, borane-ammonia (B-A) and NaOH were applied to release *O*-linked

glycans (Merry and Astrautsova, 2003). As shown in Figure 5.7.b, B-A was ineffective in the reduction of 'Big CSEP'. NaOH partially decreased its signal intensity, while blurred and diffused the lane. So it was unable to conclude if NaOH-mediated deglycosylation (*O*-linked) worked on 'Big CSEP' at this stage. The decrease of 'Big CSEP' signal can be caused by the destruction of protein backbone or other side effects of NaOH.

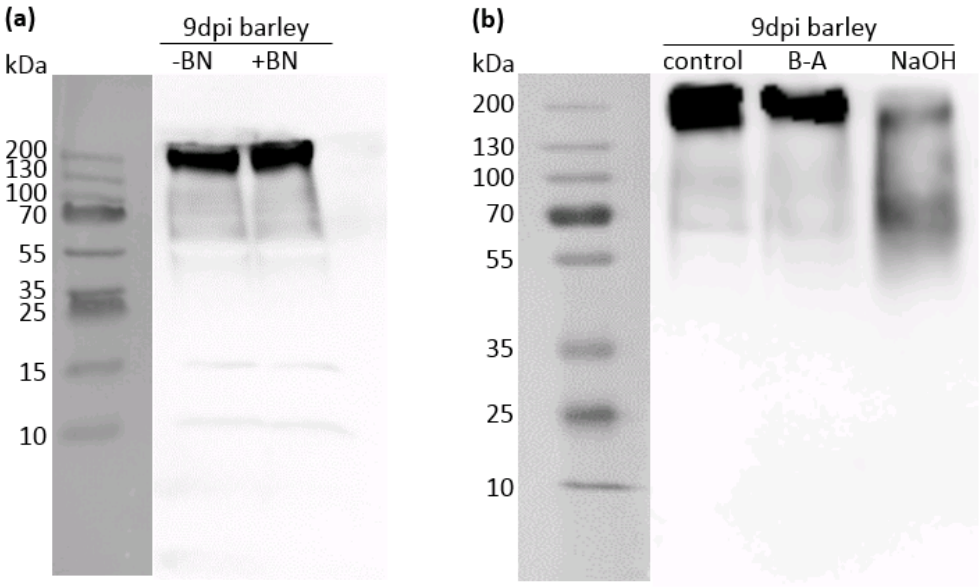

Figure 5.7 Western blot analyses of proteins after treatment with BN, B-A or NaOH (a) Total proteins (30 µg) from 9 dpi barley leaves were treated with BN and then separated on a Tris-Tricine SDS-PAGE gel, followed by a western blotting analysis (b) Proteins (20 µg) were treated with B-A or NaOH separately and then separated on a Tris-Glycine SDS-PAGE gel before continuing with immunoblotting. One negative control (untreated) was loaded on each gel. 'Big CSEP' appeared in BN and B-A treated samples. But its signal significantly reduced and blurred after incubation with NaOH.

Apart from using chemicals to cleave *O*-glycans from proteins, a typical *N*-glycosidase (PNGase F) was used to release enzymatically any potential *N*-glycans from the 'Big CSEP'.

Proteins (9 dpi) was treated with PNGase F and visualized by western blot to show if the 'Big CSEP' had been affected. Together with this treated sample, two negative controls were included: untreated 9 dpi and PNGase F alone. Single *N*-linked glycoprotein RNase B was used as a positive to assess the deglycosylation efficiency caused by PNGase F. The SDS-PAGE gel shift (see Figure 5.8.b) indicated that PNGase F effectively released *N*-glycans from a bona fide glycoprotein. As the 'Big CSEP' was not affected by this treatment (Figure 5.8.a), I do not consider it to be an *N*-linked glycoprotein. Additionally, I also used NetNGlyc 1.0 Server to predict the potential *N*-glycosylation sites of CSEP0064 and found no positive results.

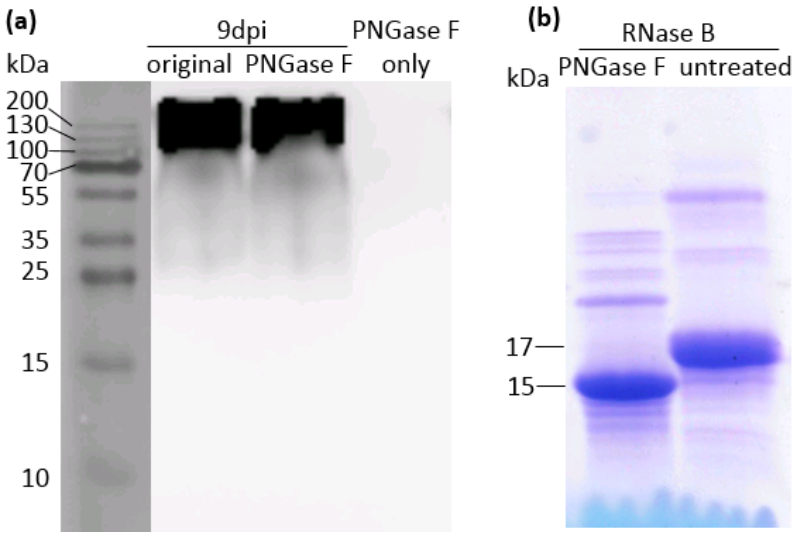

Figure 5.8 Western blot analysis and Coomassie Blue stained SDS-PAGE gel of proteins +/- treated with PNGase F. (a) 9 dpi samples -/+ treated with PNGase F (lane 1 and 2) and a negative control (enzyme alone, lane 3) were separated on an SDS-PAGE gel and assessed by western blotting. The treated sample showed an intensive band signal ~ 200 kDa as the same as untreated one. (b) Successful deglycosylation of RNase B was visualized by Coomassie Blue stained SDS-PAGE gel. The upper band ~ 17 kDa and lower band ~ 15 kDa represent the intact and deglycosylated forms of RNase B, respectively.

PNGase F was limited in cleaving *N*-glycans and NaOH seemed to destroy the whole protein backbone rather than selectively remove *O*-glycans. Finally, TFMS was used to remove all *N*-

linked, *O*-linked and glycosaminoglycans while retaining the intact polypeptides (Edge, 2003). Proteins were precipitated and then dried thoroughly to provide an anhydrous condition for TFMS deglycosylation. Anisole was added to achieve a final concentration of 10% in TFMS. This was used as a scavenger to prevent any secondary reactions between amino acid side chains (P.Bahl, 1987). In Figure 5.9, it was clear that the 'Big CSEP' signal had been completely removed with TFMS treatment. The same sample stained by Coomassie Blue on SDS-PAGE gel showed multiple clear and intact protein bands, suggesting that the removal of 'Big CSEP' was not caused by complete destruction of proteins but selectively (Figure 5.9.b). A slight shift of the band around 55 kDa (asterisk) was observed in the treated sample.

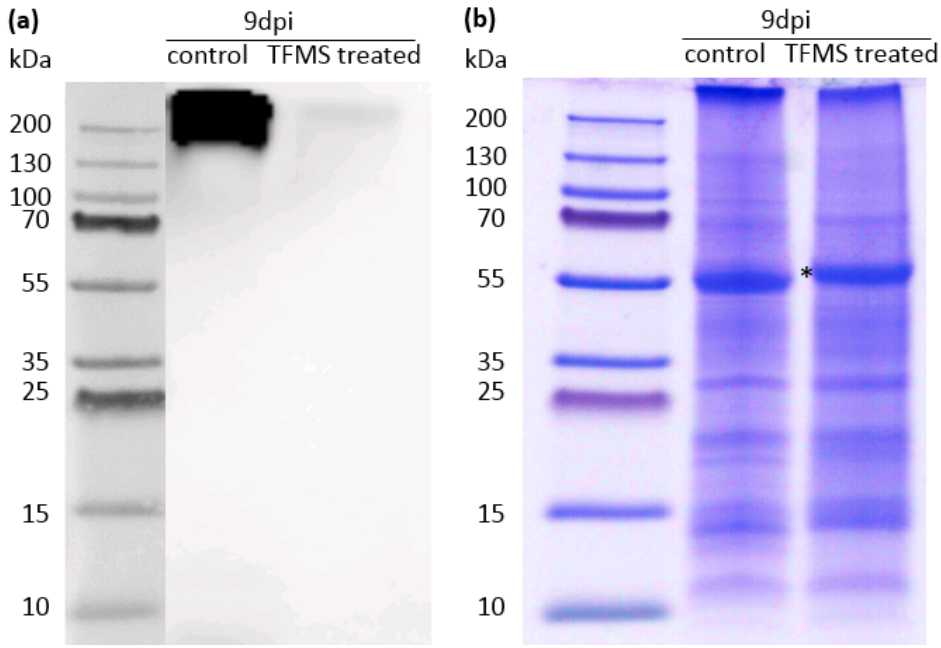

Figure 5.9 Western blot analysis of 9 dpi barley proteins +/- treated with TFMS. (a) Nine dpi samples -/+ treated with TFMS (lane 1 and 2) were separated on an SDS-PAGE gel and analyzed by western blotting. 'Big CSEP' signal disappeared in treated sample. (b) Same samples were loaded onto another gel and stained using Coomassie Blue to show the protein backbones after treatment. Most (but not all) of the protein bands stayed the same. The band a shifted up in treated sample (shown by an asterisk).

5.4.3 Digestion and identification of 'Big CSEP'

An MRM-MS approach was used to further identify the 'Big CSEP'. To isolate the 'Big CSEP' complex from total protein extracts, immunoprecipitation using anti-CSEP0064 antibody was attempted first. However, without a suitable primary antibody which can replace anti-CSEP0064 in subsequential western blot analysis of immunoprecipitated proteins, anti-CSEP0064 had to be used twice during the whole procedure. In this case, the secondary antibody used in western blot can not only detect primary antibody-binding antigen, but also primary antibody eluted during immunoprecipitation. This means the co-elution of primary antibody was particularly problematic. The contaminating bands from the co-eluted antibody masked the region where the 'Big CSEP' should be (Supplementary Figure 3.a). A crosslinker disuccinimidyl suberate (DSS) was used to prevent the co-elution of antibodies but did not work as expected (Supplementary Figure 3.b).

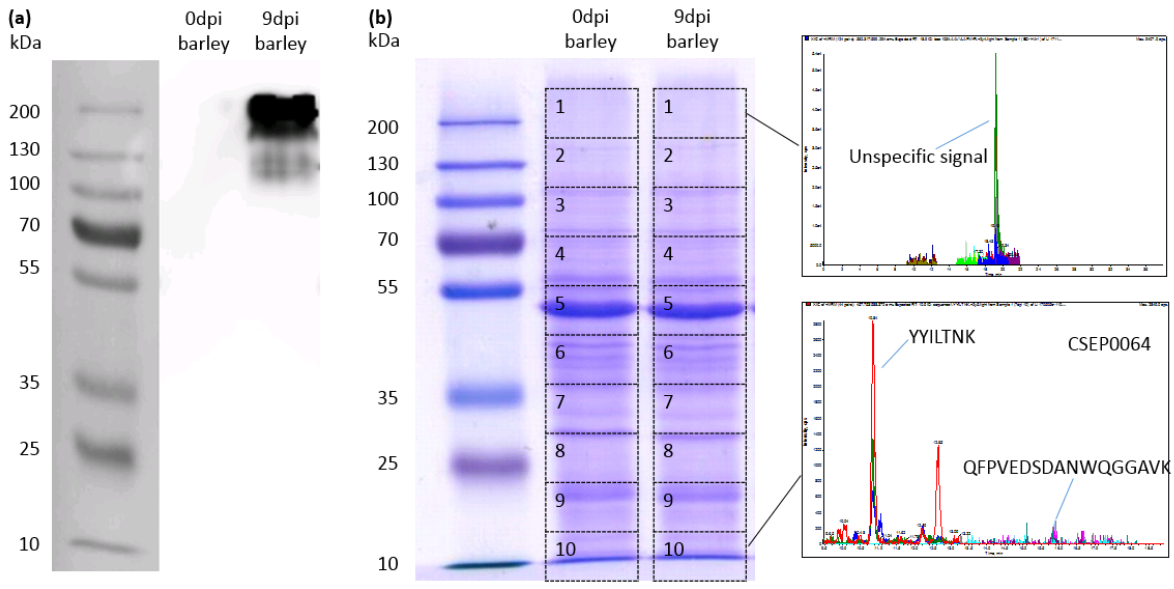

Figure 5.10 MRM-MS of proteins digested in-gel using trypsin. (a) Western blot analysis of 0 dpi and 9 dpi extracts to show the position of 'Big CSEP'. (b) Samples were separated by SDS-PAGE and stained with Coomassie Blue. Both lanes of 0 dpi and 9 dpi samples were then excised and cut into ten small pieces separately and numbered from #1 to #10. Proteins in those twenty pieces were digested in gel by trypsin and then analyzed by MRM-MS. Target peptides

of CSEP0064 were detected in piece 10 of 9 dpi sample only. Nothing specific was detected in the gel containing 'Big CSEP'.

As I failed to isolate 'Big CSEP' through immunoprecipitation, I then excised the bands from SDS-PAGE gel and performed in-gel digestions of these bands. The whole lane of 9 dpi sample was cut into ten pieces and numbered from top to bottom. Gel piece #1 contained high molecular weight proteins around 200 kDa, including 'Big CSEP'. And piece #10 excised around 10 kDa should contain the wild-type, unmodified, CSEP0064. One 0 dpi sample was analyzed the same way to exclude any false-positive results. After MRM-MS of these samples, target peptides of CSEP0064 (YYILTNK and QFPVEDSDANWQGGAVK) were detected in gel piece #10 of 9 dpi corresponding to unmodified wild-type CSEP0064 (Figure 5.10.b). There was only an unspecific signal detected in gel piece #1 of 9 dpi, suggesting that either there is no CSEP0064 in the 'Big CSEP' band or CSEP0064 was modified and could not be detected MRM-MS.

The contamination of other high molecular weight proteins needed to be excluded to optimize the sensitivity and accuracy of MRM-MS. 'Big CSEP' was found to be relatively resistant to trypsin during in-solution digestion of total proteins (data not shown here). Accordingly, a dilution series of trypsin was carried out to find conditions which can digest all (or most of) other proteins while leaving 'Big CSEP' unaffected. The ratio of enzyme to substrate in solution digestion was recommended to be 1:100 to 1:20 (w/w) (Picotti and Aebersold, 2012). In my experiment, ratios of 0 (no trypsin), 1:400, 1:200, 1:100, 1:50, 1:25 were included. The results (Figure 5.11) showed that trypsin is effective in digesting most of the proteins even with the smallest quantity, whereas the 'Big CSEP' could only be partially digested with large amounts of trypsin. The more trypsin added the more intense the band of degraded trypsin on stained SDS-PAGE gel (asterisk in Figure 5.11.b).

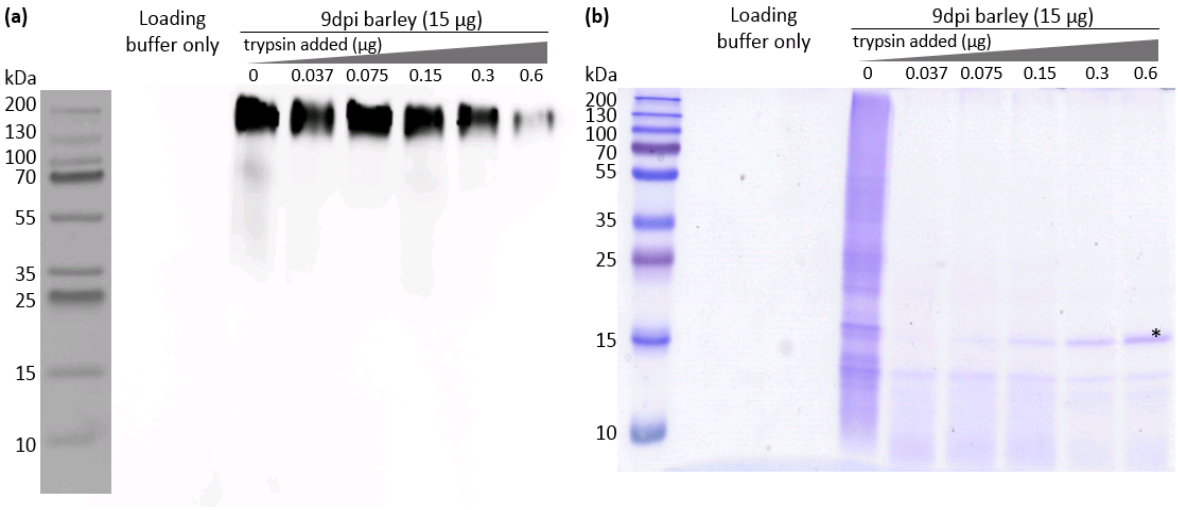

Figure 5.11 Western blotting of 9 dpi barley samples digested with increasing amount of trypsin. Increasing amount of trypsin (0 µg, 0.037 µg, 0.075 µg, 0.15 µg, 0.3 µg and 0.6 µg) was added to six samples, separately. All digestions started with the same amount (15 µg) of 9 dpi total proteins and then separated by SDS-PAGE. (a) Immunoblotting analysis to determine if the 'Big CSEP' has been digested. (b) Proteins were stained with Coomassie Blue. Most of the bands disappeared in lane labeled 0.037 µg, suggesting that trypsin added in this sample was enough for digesting almost all proteins. The same sample in western blot result (a) showed an intensive signal of 'Big CSEP'. The band around 15 kDa (asterisk) represents degraded trypsin.

With this approach to 'extract Big CSEP' from total plant extracts by digesting other proteins, I was able to cut relatively clear gel piece (top of the gel where 'Big CSEP' appeared) and perform in-gel digestion of this piece. Trypsin was added to 9 dpi sample in low ratio of 1:300 and 1:100, respectively. Two treatments were included half for testing the reproducibility of previous experiment and half for choosing the best sample after digestion. As shown in Figure 5.12, both treatments can remove most of the proteins with 'Big CSEP' remain. But sample with a ratio of enzyme to substrate in 1:100 showed clearer lane, which indicated fewer contaminations in target area (labelled with a red frame). However, the CSEP0064 signal was still non-detectable in this area.

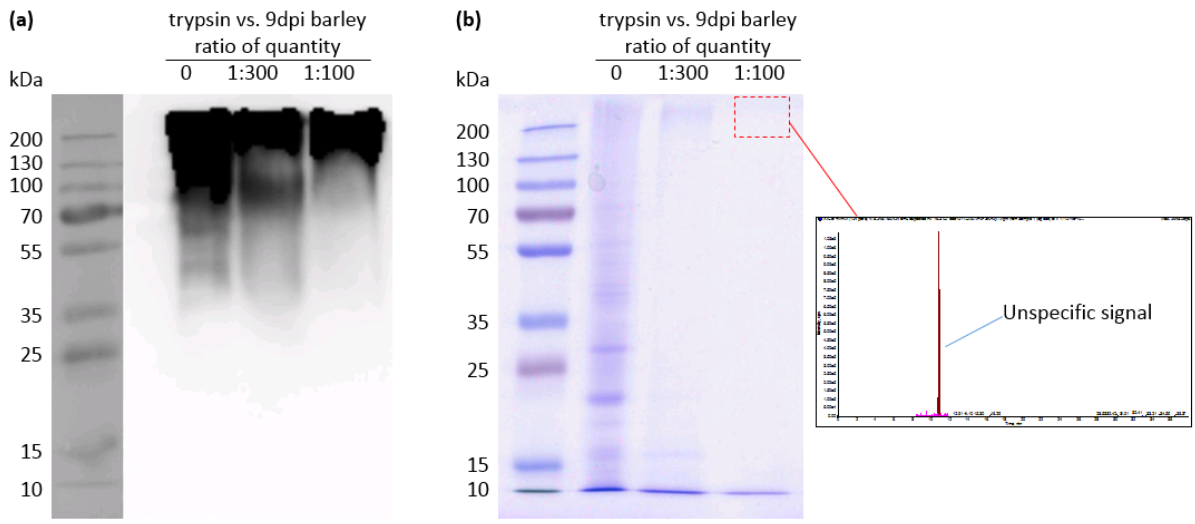

Figure 5.12 Western blot analysis of digested proteins and MRM-MS of those proteins which have been further digested in-gel. Total proteins from 9 dpi barley were extracted and treated with small amount of trypsin to substrate: enzyme ratios of 300:1 and 400:1, respectively. (a) Analysed by western blotting. Both samples digested with trypsin showed obvious 'Big CSEP' signals. (b) Proteins were then stained and digested in-gel, followed by MRM-MS. One unspecific signal was detected in 'Big CSEP' gel piece.

5.5 Discussion

The initial objective of this study was using western blot to investigate the expression of CSEP0064 in infected host tissues. Unlike previous studies which quantified the mRNA transcript levels of CSEPs, I investigated the abundance of CSEP0064 protein. Even through the western blot approach is well established, many problems can arise for different reasons, including size and complexity of target proteins (Bass et al., 2017, Gallagher et al., 2008, Sanders et al., 2016). As *B. graminis* f. sp. *hordei* is an obligate biotroph, the protein extracts need to be obtained from infected plants. Here I showed that the loadings of total proteins need to be large enough (≥ 30 μg) to allow the detection of CSEP0064 by western blot. Given that CSEP0064 is a small molecular mass protein (10.8 kDa), Tris-Tricine SDS-PAGE gel provided better resolution of CSEP0064. This is in agreement with other studies (Bindschedler et al.,

2011, Schagger, 2006). Apart from these, TCA/Acetone precipitation led to higher solubility of total proteins, which can be visualized by Coomassie Blue staining of proteins. For example, the large subunit of RuBisco was clearly visible as an intensive band around 55 kDa in TCA precipitated samples. Based on the optimizations of western blot, I observed that CSEP0064 was only detectable in heavily infected barley leaves (earliest at 7dpi), suggesting that the biomass of fungus at late stage of infection was sufficient for detecting this effector in host plant. This is consistent with previous MS-based *in planta* proteome analyses of *B. graminis* f. sp. *hordei,* where 7-10 dpi infected barley leaves were used (Bindschedler et al., 2009). However, quantitative results based on RT-qPCR showed that CSEP0064 increases in expression at early stage, particularly the initial haustorial formation of *B. graminis* f. sp. *hordei* infection (Pennington et al., 2015, Pliego et al., 2013). The discrepancy between previous qPCR results and my western blot results revealed the poor mRNA-protein correlation in these complex biological samples, which in turn highlighted the importance of investigating protein abundance to understand *B. graminis* f. sp. *hordei*-barley interactions.

I also detected a prominent band representing CSEP0064 in isolated haustoria, but not other dissected structures. It further proved that this effector is predominantly expressed in the feeding structure of fungus (Pedersen et al., 2012). As shown in chapter 6, a portion of the isolated haustoria was still surrounded by extrahaustorial membrane, meaning that the HA protein extracts also included proteins that accumulate in extrahaustorial matrix. Considering the fact that extrahaustorial matrix is considered to be a key battleground of fungal effectors and plant-secreted molecules during *B. graminis* f. sp. *hordei*-barley interactions (Pedersen et al., 2012, Lo Presti et al., 2015), CSEP0064 in haustoria and extrahaustorial matrix could both contribute to the abundant signal detected in HA sample. One of the main issues while using western blot is detecting unexpected bands (Bass et al., 2017, Hilgarth and Sarge, 2005). In this study, I observed three unexpected bands in HA samples. Two bands sized just above 25

kDa and 35 kDa were considered to be the dimer and trimer of CSEP0064, and the band around 15 kDa may be an unusual CSEP0064 conjugate. Identification of these bands can be achieved by in-gel digestion and MRM-MS, which would help to understand the process of effectors in haustoria and extrahaustorial matrix.

One intriguing and important observation of this study is that I detected an unexpected intense, large and diffuse signal in infected barley leaves. This band migrated with an apparent molecular mass around 200 kDa, i.e., much higher than CSEP0064. Additionally, this signal was specifically recognized by anti-CSEP0064 antibody and remained detectable while using purified antibody which had been immunoprecipitated with recombinant CSEP0064. It was therefore named 'Big CSEP' and considered to be relevant to CSEP0064. Compared to CSEP0064, the 'Big CSEP' accumulated to detection level in barley leaves much earlier and was visible already three days after inoculation. Moreover, it was also detected in epiphytic mycelia as well as in haustoria-containing epidermis. These findings appear to contrast with those previously reported by Bindschedler *et al*. (2011). That study compared the proteomes of sporulating hyphae and haustoria that dissected from 7-10 dpi barley and found CSEP0064 (together with other 70 proteins) to be haustoria exclusive (Bindschedler et al., 2011). An explanation of this discrepancy could be that the method used to identify protein from MS data eliminated modified peptides. I failed to observe CSEP0064 specific peptides in the MRM-MS analysis of 'Big CSEP' gel slice (see Figure 5.10&12) for the same reason.

The most critical step was characterization of the 'Big CSEP'. Assuming that 'Big CSEP' is related to CSEP0064, I tested if it was a complex of unknown molecules-binding CSEP0064. Pliego *et al.* (2013) suggested that CSEP0064 is an RNase-like effector and its contribution to fungal infection might relate to host RNA binding. Treatment with RNase A or Benzonase Nuclease failed to have any visible effect on the electrophoretic mobility or the intensity of the band, though these enzymes have been confirmed to efficiently remove contaminating RNAs

from RNA-binding protein in various studies (Kang et al., 2007, Duss et al., 2014, Francisco-Velilla et al., 2016). I next speculated that the 'Big CSEP' is host protein-binding CSEP0064. Co-immunoprecipitation (Co-IP) has been used as an efficient way to identify protein-protein interactions by using antibodies specific to a target protein to capture proteins binding to the target protein. This technique has been used with success in pathogen-host interactions to identify the host targets of effector proteins (Yang et al., 2017, Katagiri et al., 2002, Smaczniak et al., 2012). Four putative interactors of CSEP0064 have been reported through Y2H screening (Pennington et al., 2016). However, without antibodies for any of these putative interactors of CSEP0064, I was unable to perform a complete Co-IP to investigate this hypothesis. I failed to detect 'Big CSEP' in immunoprecipitated complex using western blotting for the same reason.

PTMs of protein can cause an electrophoretic mobility shift of proteins (Leach and Brown, 2012, Salomon and Orth, 2013). For example, phosphorylation of a protein kinase mTOR increased its size from 200 kDa to 289 kDa (Kinoshita et al., 2009), SUMOylation of a heat shock protein in *Arabidopsis* shifted the molecular weight from 18 kDa to 100 kDa (Reut Cohen-Peer, 2010) and *N*-glycosylated Slp1 (a rice blast effector) was 40 kDa bigger in molecular mass than wild-type (Mach, 2014). Among them, *N*-linked glycosylation has been described in different plant-pathogenic fungal effectors and found to influence plant immune system (Fang et al., 2016, Hirai et al., 2011, Chen et al., 2014a). For deglycosylation analysis, all these studies used Endoglycosidase H (Endo H) or Peptide-N-Glycosidase F (PNGase F), or both, to cleave *N*-glycans from target protein enzymatically. Here, PNGase F did not affect 'Big CSEP' mobility or intensity. This is consistent with the absence of predicted *N*-glycosylation site of CSEP0064 on NetNGlyc 1.0. In contrast, TFMS, which has been used extensively to remove *N*-linked, *O*-linked and glycosaminoglycan chains by cleaving linkages between sugars (P.Bahl, 1987, Edge, 2003, Maddi et al., 2009, Murakami et al., 2014), abolished the 'Big CSEP' signal completely. I therefore propose that, 'Big CSEP', could be a

conjugate of CSEP0064 modified by *O*-glycosylation or a combination of glycosylation variants. This also suggested that the signal decrease caused by NaOH was the result of selective cleavage of *O*-glycans. The challenge here, is that without detecting a single band (at expected size) representing deglycosylated-CSEP0064 in TFMS-treated sample, I cannot exclude the possibility of removing 'Big CSEP' by other side-effects of the harsh TFMS reagent, leading either to degradation of the complex, or modifications of the antigen that made it no longer recognised by the antibody. However, published studies highlight the advantage of using TFMS is cleaving all types of carbohydrate without breaking peptide bond (Murakami et al., 2014, Merry and Astrautsova, 2003, Takahashi et al., 2009). Due to time limitations, further verification of TFMS-treated sample could not be carried out. Further tests may include one *O*-linked glycoprotein as a positive control to illustrate the efficiency/activity of TFMS in deglycosylation.

5.6 Work in progress

Based on the method I developed to digest contaminating proteins while leaving 'Big CSEP' by a small quantity of trypsin (Figure 5.11), total proteins of 0 dpi and 9 dpi will be initially digested with a small amount of trypsin (first digestion). Half of the first-digested 9 dpi sample will be kept as a control, the other half and 0 dpi sample will be processed as follows. Ultrafiltration will be performed to remove digested peptides with several repetitions. After removal of peptides, samples (theoretically only undigested 'Big CSEP' and a few contaminations) will be digested a second time with a large amount of trypsin to digest 'Big CSEP' completely (second digestion). Digested products from each step will be examined by western blotting to confirm the 'appearance' and 'disappearance' of 'Big CSEP'. Finally, three samples, first-digested 9 dpi, second-digested 0 dpi and 9 dpi will be sent for MS. Ideally, target peptides of CSEP0064 will be detected in both 9 dpi samples. Additionally, MS can help us to understand what else exists in 'Big CSEP'.

Preliminary tests of experimental design expect MS have been done by myself. Final experiments and MS will be performed by our collaborators Laurence Bindschedler and Sebastien Lambertucci at RHUL. They are planning to carry out MS analyses of TFMS-treated and TFMS-untreated samples as well.

5.7 Conclusions

In conclusion, *B. graminis* f. sp. *hordei* effector CSEP0064 (10.8 kDa) is detectable by western blotting in heavily infected barley leaves and isolated haustoria. Unexpectedly, a protein complex shown as a diffuse signal around 200 kDa, possibly related to this effector is observed in all fungal structure-containing materials. This 'Big CSEP' can be partially digested with trypsin and completely removed by TFMS, suggesting it is a glycosylated form of CSEP0064. The first challenge for future work is to test whether CSEP0064 really is glycosylated and determine the functional significance of this.

6 Detection and quantification of CSEP0064 using MRM-MS

6.1 Introduction

Traditionally, the proteome investigations rely on antibody-based approaches such as western blot and immunofluorescence (Maiolica et al., 2012, Gallagher et al., 2008). However, the detection and quantification of proteins using antibody-based method has limitations. For example, the assay performance depends on a large degree on the performance of antibodies (Gingras et al., 2007, Chen et al., 2013). Mass spectrometry (MS), which measures the mass-to-charge ratio (m/z) of a molecule, can overcome many of the limitations of affinity-based approaches, has emerged as an alternative method in proteomic studies (Walther and Mann, 2010, Zauber et al., 2013). Over the past two decades, MS-based methods has been routinely used for the detection and quantification of proteins, as well as identification of post-translational modifications, particularly in clinical studies (Liu et al., 2015, Kosako and Nagano, 2011). Moreover, MS-based proteomics allows the analysis of proteins across multiple highly-complex samples or under different conditions simultaneously (Chen et al., 2017, Delaunois et al., 2014). In MS-based proteomics, proteins or peptides are separated either by gel-based methods such as two-dimensional (2D)-PAGE or liquid chromatography (LC)-based (gel-free) techniques (shotgun), and subjected to MS or tandem MS (MS/MS) analysis (Figure 6.1) (Chen and Yates, 2007, Maiolica et al., 2012). However, it is worth noting that the shotgun proteomic concept has expanded. It does not exclusively refer to the gel-free strategy, but may also be coupled to 2-DE protein separation (Matallana-Surget et al., 2010, Nguyen et al., 2014).

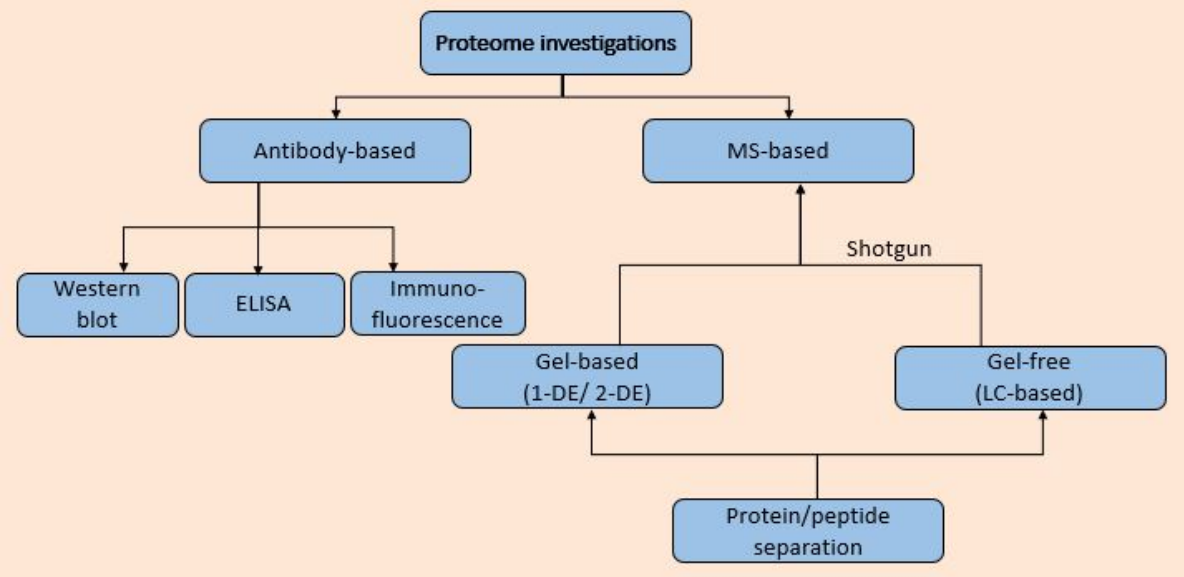

Figure 6.1 Representative approaches for proteome investigations. Antibody-based methods include western blot, immunofluorescence, enzyme-linked immunosorbent assay (ELISA) and so on. Traditionally, MS-based proteomics has been be divided into two strategies: gel-based and gel-free. The former uses classical 1-DE or 2-DE techniques coupled to MS or MS/MS, while the latter is based on LC-MS/MS as proteomic strategy and usually referred to as shotgun proteomics.

Shotgun MS is also referred to as a discovery proteomic method (Domon and Aebersold, 2010). As the most widely used MS-based approach, it allows the generation of the vast majority of proteomic data available today (Maiolica et al., 2012, Blom et al., 2004). In the context of plant-pathogen interactions, shotgun MS has been successfully employed to identify pathogens, characterize signalling factors produced by pathogens, monitor the expression of proteins upon infection and identify post-translational modifications (Gupta et al., 2015, Nat et al., 2007, Tine et al., 2005). For example, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS has been used to identify pathogens causing rust disease on wheat (Beinhauer et al., 2016) and downy mildew on lettuce (Chalupova et al., 2012) by analysing the spores. A gel-based MS method was used to compare the proteome-level changes in wheat prior and after *Puccinia triticina* infection, leading to the identification of seven up-regulated

host proteins in 9-day post infected leaves (Rampitsch et al., 2006). In addition, several host-secreted proteins including PR proteins were identified in rice apoplast during rice-*M. oryzae* interaction by 2-DE coupled with MS method (Shenton et al., 2012). Although it is suggested that identification of pathogen-secreted proteins during host-pathogen interactions using MS is more challenging due to their low abundance, some studies have successfully detected and identified pathogen effectors using shotgun MS method (Padliya and Cooper, 2006, Petre et al., 2015, Miguel et al., 2006). For instance, 24 fungal proteins were identified using LC-MS/MS in beans infected by *Uromyces appendiculatus,* while 34 fungal proteins were found in infected soybean leaves (Cooper et al., 2016).

In the particular case of *B. graminis,* proteome studies also benefit from recent advances in MS-based techniques. For example, 441, 775 and 47 proteins were identified in *B. graminis* f. sp. *hordei* conidiospores, epiphytic sporulating hyphae and haustoria-contained epidermis, respectively, using a combination of 1-DE and LC-MS/MS (Bindschedler et al., 2009). Another large-scale proteome study of *B. graminis* f. sp. *hordei* conidia was performed using 2-DE coupled with MALDI-TOF/MS and identify 123 fungal polypeptides (Noir et al., 2009). Additionally, LC-MS/MS-based proteomic analysis of isolated *B. graminis* f. sp. *hordei* haustoria, led to the identification of 204 haustoria proteins (Godfrey et al., 2009). There are several other studies listed in Table 6.1. Taken together, these large-scale proteome studies not only contribute to the protein discovery, but also enable the comparison of tissue-specific proteomes (Bindschedler et al., 2016).

Table 6.1 Recent reports on proteome studies of *B. graminis*. rpLC-nESIMS/MS means reverse phase liquid chromatography-nanoESI tandem MS.

Plant/pathogen	structures (tissues)	proteins identified	MS method	Reference
Barley/*B. graminis* f. sp. *hordei*	conidiospores	123 proteins	2-DE and MALDI-TOF/MS	(Noir et al., 2009)
Barley/*B. graminis* f. sp. *hordei*	conidia, sporulating hyphae, and haustoria inside epidermal cells	441 proteins, 775 proteins, 47 proteins	1-DE and LC-MS/MS	(Bindschedler et al., 2009)
Barley/*B. graminis* f. sp. *hordei*	isolated haustoria	204 proteins	LC-MS/MS	(Godfrey et al., 2009)
Barley/*B. graminis* f. sp. *hordei*	sporulating hyphae, epidermis containing haustoria	71 haustoria-exclusive proteins	1-DE and rpLC-nESIMS/MS	(Bindschedler et al., 2011)
Barley/*B. graminis* f. sp. *hordei*	sporulating hyphae, epidermis containing haustoria	14 proteins 15 proteins,	LC-MS/MS	(Amselem et al., 2015a)
Barley/*B. graminis* f. sp. *hordei*	epidermis containing haustoria	putative BEC1054 interactors	LC-MS/MS	(Pennington et al., 2015)
Wheat/*B. graminis* f. sp. *tritici*	infected leaf	45 proteins	2-DE and MALDI-TOF/MS	(Mandal et al., 2014)

Plant/pathogen	structures (tissues)	proteins identified	MS method	Reference
Wheat/*B. graminis* f. sp. *tritici*	wheat grains	85 proteins	2-DE and MALDI-TOF/MS	(Li et al., 2017b)
Wheat/*B. graminis* f. sp. *tritici*	infected leaf	46 proteins	2-DE and MALDI-TOF/MS	(Li et al., 2017a)

Although shotgun proteomics has also been used for protein quantification though labelling or label-free strategies, it is not the optimal method for quantitative measurement of identified proteins due to its limited sensitivity and stochastic nature (Wienkoop and Weckwerth, 2006, Gonzalez-Fernandez and Jorrin-Novo, 2012). Targeted proteomics, is emerging as an alternative approach that complements the capabilities of untargeted shotgun MS for more reliable and accurate quantification of pre-selected proteins (Liebler and Zimmerman, 2013, Lange et al., 2008b, Baker and Panisko, 2011). Nowadays, the most widely used targeted MS technique is multiple reaction monitoring (MRM), which is also called selected reaction monitoring (SRM) (Holman et al., 2012, Frank and Uwe, 2011). MRM-MS has the potential to detect and quantify predetermined proteins with high precision in complex samples by utilizing triple quadrupole (QQQ) mass spectrometers (Picotti and Aebersold, 2012). In an MRM experiment, the first (Q1) and third (Q3) quadrupoles act as filters to select predefined m/z values corresponding to the targeted peptide ion and a specific fragment ion of the peptide, whereas the fragmentation happens in the second quadrupole (Q2) (Schmidt and Urlaub, 2012, Schumacher et al., 2014, Mead et al., 2009). Such particular peptide/fragment ion pairs are termed transitions. Key steps for MRM-MS are summarized in Figure 6.2. The experiments begin with a list of target proteins, and then prior information (e.g., shotgun proteomics) is needed for selecting the suitable proteotypic peptides to represent the proteins of interest (Brownridge et al., 2011). Because MRM-MS measurements are based on monitoring the

transitions to produce quantifiable chromatographic peaks, selection of transitions is critical for MRM performance (Lange et al., 2008a, Gingras et al., 2007). Furthermore, the quantification of target peptide in an MRM-MS assay is commonly achieved by introducing an stable isotope-labelled synthetic peptide as an internal standard (Karlsson et al., 2012). As an isotopically labelled version of the target peptide, the internal standard behaves identically to the analyte itself, both in fragmentation and chromatographic retention in MS (Abbatiello et al., 2010). Thus, the transition peaks derived from the native unlabelled sample (analyte) and the internal standard can be distinguished and compared for further quantitative analysis.

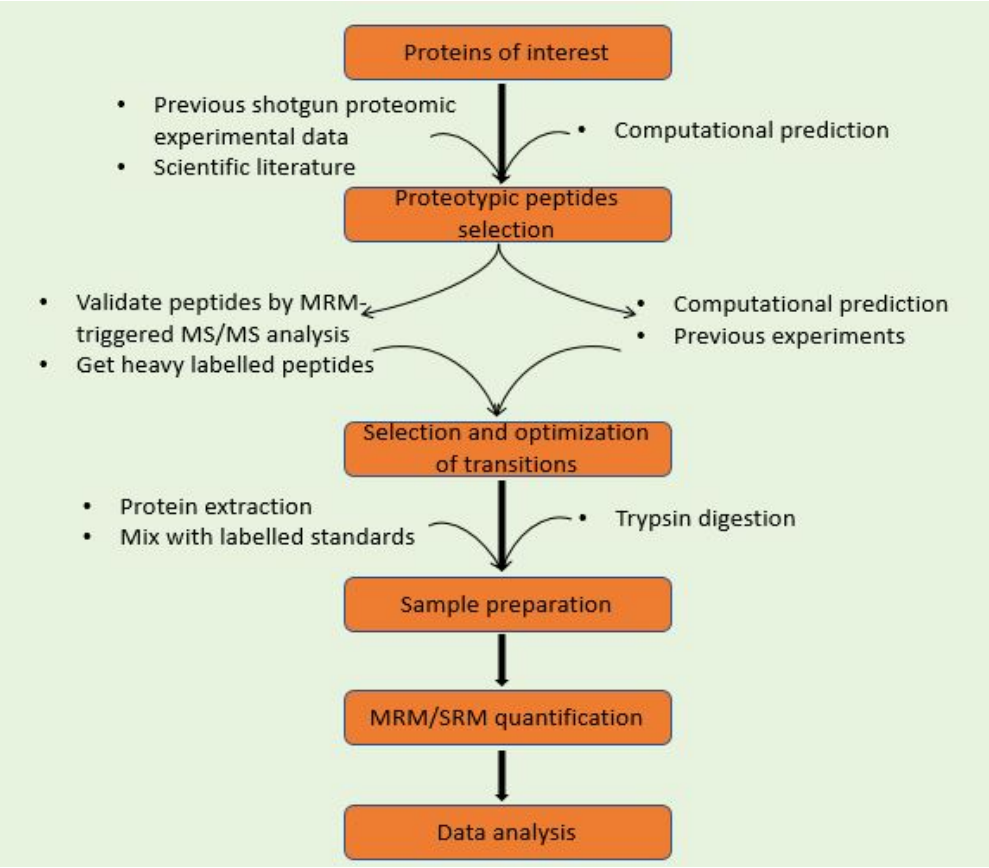

Figure 6.2 Workflow for an MRM-MS assay. See the text for discussion.

Although MRM-MS has already been routinely used in clinical researches (Wolf-Yadlin et al., 2007, Betke et al., 2014), only a few studies reported the employment of this technique for investigating plant-pathogen interactions. As an optimal approach for protein quantification,

MRM-MS has been used to analyse the quantitative changes of PR1 peptides after wounding and methyl jasmonate treatment in tomato (Chen et al., 2014b). Similarly, the fungal infection induced expression of some defense-related phytohormones were quantified using MRM-MS in *Arabidopsis* (Riet et al., 2016). Apart from the measurement of proteins at a native state, MRM-MS offers the possibility of quantification of post-translational modifications as well (Pruitt et al., 2015, Kettenbach et al., 2011). For example, the peptides of *Arabidopsis* R protein RRS1 were found to be acetylated by bacterial effector PopP2 using MS, and the relative levels of acetylation of each single peptides were quantified using MRM-MS (Sarris et al., 2015). MRM-MS was used in another study to investigate the interactions between *Arabidopsis-P. syringae* pv. *tomato* DC3000, leading to the identification of a differentially phosphorylated kinase (MKKK7), which negatively regulates the PTI signalling in *Arabidopsis* (Mithoe et al., 2016). In addition, using MRM-MS, four defense signalling-related phosphorylated proteins were quantified in soybean (Van Ness et al., 2016). However, to my knowledge, MRM-MS has not been used in *B. graminis*, not to mention the quantitative analysis of CSEPs.

In the previous chapter, I used western blot to detect *B. graminis* f. sp. *hordei* effector CSEP0064 in dissected tissues of infected barley leaves. CSEP0064 and its potential glycosylated forms were detected in epiphytic, epidermis and isolated haustoria. Due to the low abundance of CSEP0064 and the limitation on the sensitivity of western blot technique, it is still unknown if CSEP0064 is secreted into the plant cell. Therefore, in this chapter, I used MRM-MS to detect CSEP0064 in different structures to see if this technique could help to elucidate the partitioning and quantitative levels of *B. graminis* f. sp. *hordei* effectors in infected tissues.

6.2 Objectives

1. To utilize MRM-MS to detect CSEP0064 in different dissected tissues of infected barley leaves.

2. To use MRM-MS to quantify the expression levels of CSEP0064 in different structures of infected leaves.

3. To further investigate the potential modification of CSEP0064 at peptide level.

6.3 Materials and Methods

6.3.1 Plant and fungal materials

6.3.1.1 Dissection of infected barley

The growth conditions of growing barley seedlings and *B. graminis* f. sp. *hordei* have been described in 4.3.1. The first leaves of 7dpi (when they are heavily infected) plants were used for dissection. To collect epiphytic sporulating hyphae, the leaves were immersed in 5% cellulose acetate acetone and then placed onto two Pasteur pipettes. After the acetone evaporated, the cellulose acetate membrane with the epiphytic fungal structures (sample EP) embedded in it was peeled away from the rest of the leaf. Following this, the abaxial epidermis (ED) which contains haustoria was carefully stripped using forceps. EP and ED samples were snap-froze with liquid nitrogen and stored at - 80 ℃ freezer till protein extraction. Roughly 80 leaves were dissected per individual experiment and three independent experiments were performed.

6.3.1.2 Isolation of haustoria

To isolate haustoria, the fresh epidermis was floated in 15 ml of 10 mM MES hydrate buffer (pH 5.3) directly after peeling. Once sufficient epidermal strips were collected (~80 leaves), the strips were transferred to 2% (w/v) cellulase Onozuka R-10 (Duchefa Biochemie,

Netherlands) in MES buffer in a 50 ml Falcon tube. The mixture was then incubated at 28 ℃ with gentle shaking (e.g. 80 rpm on a rotating platform) for two hours. After incubation, the buffer was slowly poured and filtered through a 70 μm nylon mesh sieve (ThermoFisher Scientific, USA) into a new 50 ml tube. A pair of forceps was used as a barrier to retain larger pieces of undigested plant material while pouring. Once complete, the buffer was centrifuged at 3270 *g* for 10 min at 4 ℃. After the centrifugation, the isolated haustoria (HA) were contained in the pellet and the supernatant contained the molecules from host cytoplasm, which was defined as epidermis subtract haustoria (ED-HA) here. ED-HA samples were used for protein extraction immediately, whereas HA samples could be either stored at -80 ℃ till needed or stained immediately for quality control. For protein extraction, three independent experiments were conducted for collecting HA and ED-HA samples.

6.3.1.3 Haustoria staining

To estimate the quality of isolated haustoria, one additional HA sample was needed for staining. The haustorial pellet was resuspended in 300 μl PBS buffer (pH 7.4). Then wheat germ agglutinin (WGA)-Alexa Fluor 488 (ThermoFisher Scientific, USA) was added into the suspension to a final concentration of 10 μg/ml and mixed well by inverting the tube. To visualize the stained haustoria, the mixture was loaded onto a microscope glass slide and observed under the GFP filter with an epifluorescence microscope (ZEISS, Germany).

6.3.2 Protein sample preparation

6.3.2.1 Protein extraction for EP and ED

The cellulose acetate peels and epidermal strips were ground in liquid nitrogen (with a little sand for epiphytic materials) to fine powder, and then subjected to TCA/Acetone protein precipitation (4.3.2.2).

6.3.2.2 Protein extraction for HA

To extract proteins from haustorial pellet, 100 µl of denaturing extraction buffer (4.3.2.2.) was added and mixed with the pellet. The mixture was transferred into a 1.5 ml Eppendorf tube, vortexed thoroughly and then incubated on ice for 10 min, followed by 20-min centrifugation at 13,000 *g* on a table-top Eppendorf microcentrifuge at 4 ℃. Then the supernatant was carefully transferred to a new 1.5 ml tube.

6.3.2.3 Ethanol precipitation for ED-HA proteins

To precipitate proteins, 9 volumes of pre-cooled (-20 ℃) ethanol was added to the saved supernatant from 5.3.1.2. The mixture was incubated at -20 ℃ overnight. On the next day, the proteins were separated from the soluble fraction by centrifugation at 3270 *g* for 20 min at 4 ℃. After carefully discarded the supernatant, 1 ml of cold (-20 ℃) ethanol (90%) was added to the pellet and mixed well. Then the mixture was transferred to a 1.5 ml Eppendorf tube and centrifuged at 13,000 *g* for 20 min at 4 ℃. All liquid was removed and the pellet was allowed to stand on the bench for at least 5 min to let the ethanol evaporate. Pellet was then dissolved in denaturing extraction buffer. A final centrifugation step was needed to separate any insoluble residues from soluble proteins.

6.3.2.4 Bacterial protein extraction

The bacterial pellet (from 5.3.4.3) was suspended in 1 ml of denaturing extraction buffer. Roughly 10 glass beads (Sigma-Aldrich, USA) was added to the cell suspension. Then the mixture was incubated on ice for 15 min, vortexing every 5 min, followed by 20-min centrifugation at 13,000 *g* at 4 ℃. Once completed, the supernatant was carefully transferred to a new 1.5 ml Eppendorf tube.

Protein concentration of all samples was estimated by Bradford assay (Bio-Rad, USA). A dilution series of bovine serum albumin (BSA) was included to make the standard curve for protein concentration measurement.

6.3.3 Design and construction of QconCAT plasmid

The quantification concatemer (QconCAT) technique was used for protein quantification (Pratt et al., 2006).

6.3.3.1 Selection of target proteins, peptides and transitions

Apart from CSEP0064, PR5, which is the putative interactor of CSEP0064 was also included. A list of candidate housekeeping proteins were chosen based on previous qPCR experiments (Pennington et al., 2015). Finally, two of them were selected to be used as reference proteins in MRM-MS experiments by *in silico* analysis. *In silico* trypsin digestion was performed using Skyline to predict the proteotypic peptides and transitions. Final peptide selection was based on several favourable characteristics, for example, absence of residues that with possible artefactual modification, absence of cysteine and methionine and producing multiple co-eluting transitions (Chen et al., 2017, Lange et al., 2008b). Then the identity of candidate peptides was confirmed by Enhanced Product Ion (EPI) scans. In addition, the uniqueness of candidate peptides was check by filtering against the relevant background proteome. Three to five transitions were selected per peptide. Target proteins and signature peptides are listed in the table below.

Table 6.2 Target proteins and proteotypic peptides. GAPDH represents glyceraldehyde-3-phophate dehydrogenase and TLP represents thaumatin-like protein.

Proteins	Genebank ID	Proteotypic peptide	Number of transitions
CSEP0064	CCU83233	AAVVLAFNYR	4
		GSYLEVFSSVGSGNK	4
		YYILTNK	3
GAPDH (*B. graminis* f. sp. *hordei* DH14)	CCU80715.1	TAAQNIIPSSTGAAK	4
		VIPALNGK	4
		AGISLNDNFVK	5
		VIDLISYIAGK	4
GAPDH (*H. vulgare*)	CAA42901.1	IGINGFGR	3
		AASFNIIPSSTGAAK	3
		VLPELNGK	3
		VVDLIR	4
PR5 (TLP5)	KP293850.1	SYTVWPGALPGGGVR	4
		LDPGQSWALNMPAGTAGAR	4
		CITGDCNGVLACR	4
		FGGDTYCCR	4

6.3.3.2 Construction of QconCAT plasmid

The sequences of selected peptides were randomly concatenated *in silico* and used to design the synthetic gene. The codon-optimized QconCAT plasmid (Figure 6.3) was synthesized and

subcloned into the expression vector pET100/D-TOPO by GeneArt (ThermoFisher Scientific, USA).

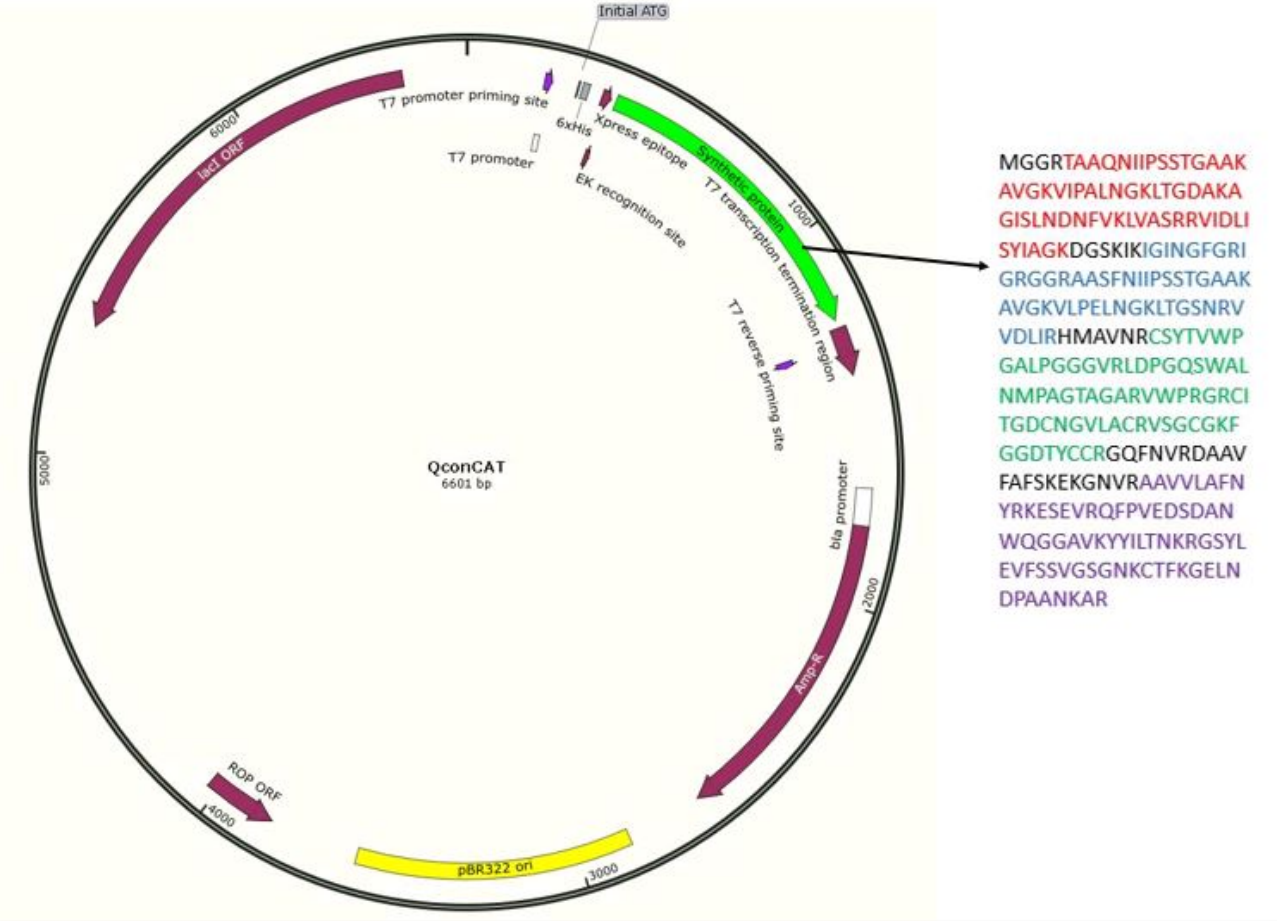

Figure 6.3 Plasmid map and the sequence of synthetic protein. Designed QconCAT was in a pET100/D-TOPO vector with a T7 promoter and an ampicillin resistance gene. Sequences in red, blue, green and purple are for GAPDH (*B. graminis* f. sp. *hordei* DH14), GAPDH (*H. vulgare*), PR5 and CSEP0064, respectively.

6.3.4 Expression of QconCAT protein

6.3.4.1 Preparation of competent E. coli *BL-21 cells*

The *E. coli* strain BL-21 was grown in 10 ml of LB medium at 37 ℃ overnight, shaking (250 rpm). On the second day, 100 µl of the overnight culture was added to 100 ml LB and incubated at 37 ℃ until the culture reached an OD_{600} = 0.3-0.5. The culture was chilled on ice for 20 min,

followed by 10-min centrifugation at 3270 *g* at 4 ℃. The bacterial pellet was then suspended in 10 ml of ice-cold 100 mM $CaCl_2$ and incubated on ice for 20 min. The cell suspension was centrifuged for 10 min at 3270 *g*, and then resuspended in 10 ml of ice-cold 50 mM $CaCl_2$ with 15 % glycerol. After another 10-min centrifugation, 0.5 ml of 50 mM $CaCl_2$ with 15 % glycerol was added to the cells and mixed well. Finally, the suspension was distributed in 100 µl aliquots and snap-frozen in liquid nitrogen. The competent cells were kept at - 80 ℃ till needed.

6.3.4.2 Heat-shock transformation

The competent *E. coli* BL-21 cells were thawed on ice for 20-30 min then mixed with 1 µl QconCAT plasmid (5 ng) by flicking the tube. The mixture was incubated on ice for another 20-30 min. Heat shock was carried out at 42 ℃ for exactly 30 s followed by quenching on ice immediately for another 2 min. Then 250 µl of SOB media was added to the cells and incubated at 37 ℃ for 60 min. Once recovery completed, 100 µl of the mixture was plated onto an LB plate with 100 µg/ml ampicillin, and incubated overnight at 37 ℃.

6.3.4.3 Expression and labelling of QconCAT protein

The expression induction of QconCAT protein in BL-21 was triggered by the addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG). Heavy isotope-labelled QconCAT peptides were used as internal standards. To achieve this, 20 amino acids (include $^{13}C_6{}^{15}N_4$ arginine and $^{13}C_6{}^{15}N_2$ lysine) were prepared into 4 solutions based on the solubility (Supplementary Table 1).

Ten ml of expression media (1x Gutnick media [33.79 mM KH_2PO_4, 77.51 mM K_2HPO_4, 5.74 mM K_2SO_4, 0.41 mM $MgSO_4{\cdot}7H_2O$], 0.4% glucose, 10 mM NH_4Cl, 1x trace element stock (Supplementary) and 1 mM of each amino acid from 4 stock solutions) was inoculated with a single colony (fresh transformant) as the starting culture and incubated at 37 ℃ overnight with 100 µg/ml of carbenicillin with shaking. On the second day, 2 ml of the overnight culture

was added to 100 ml expression media and incubated at 37 ℃ with shaking. When the OD_{600} reached 0.4-0.6, the bacterial culture was moved into a 30 ℃ incubator to slow down the exponential growth for 30 min. Once incubation finished, 1 ml of the total culture was removed and kept as uninduced sample (control), and the rest was induced by adding IPTG to a final concentration of 0.5 mM and incubated for 4 h at 30 ℃ while shaking. Finally, the culture was centrifuged at 3270 *g* for 20 min at room temperature and subjected to protein extraction.

The expression and labelling of QconCAT protein was examined by SDS-PAGE and trial MRM-MS runs.

6.3.5 In-solution protein digestion

6.3.5.1 Standard protocol for samples except HA and ED-HA

All protein samples were adjusted to final concentrations of 5 mg/ml for easier use. Prior to in-solution digestion, 9 µl of each protein extracts (analytes) were mixed with 1 µl of bacterial proteins that contains heavy-isotope labelled QconCAT proteins (IS). Then 2 µl 500 mM ABC with 100 mM DTT and 8 µl H_2O were added to the protein mixture, followed by incubation at 42 ℃ for 30 min. Once completed, 5 µl of 50mg/ml iodoacetamide was added to alkylate cysteines for 20-30 min in darkness at room temperature. Then 5 µl 100 mM DTT was added to quench the reaction. Subsequently, 40 µl 100 mM ABC was added to dilute the urea in the mixture to a final concentration of 1 M, and at the same time adjust the pH ≈ 8 (checked by pH indicator strip). Proteins were then digested with 5 µl of 200 ng/ µl sequencing grade trypsin (Sigma-Aldrich, USA) at 37 ℃ for 2 h, and for another 2 h after an additional 5 µl trypsin was supplemented. After total 4 hours incubation, the mixture was left at room temperature overnight to prolong the digestion, stopped by adding 2.5 µl of 5% FA to turn the solution acidic (checked by pH indicator strip). Finally, 1.7 µl ACN was added (final concentration of

2%) to help to keep peptides in solution and a 10-min centrifugation at 13,000 *g* was performed to remove any insoluble particulates.

6.3.5.2 Modified protocol for HA and ED-HA samples

To digest proteins from HA and ED-HA, a few modifications were included. The samples were mixed with QconCAT proteins as above. The reduction was carried out at 56 ℃ for 30 min after the addition of 1.1 µl 500 mM ABC with 100 mM DTT. Once finished, 8.9 µl H_2O was added to the sample. Then the latter steps were performed the same as standard protocol.

6.3.6 MRM-MS

Digested samples were analyzed on a 6500 QTrap hybrid linear ion trap triple quadrupole mass spectrometer (Applied Biosystems, USA). The 6500 QTrap system, which was equipped with an Ion Drive Turbo V source, was operated at positive-ion mode. Typically 40 µl injections were used for analysis. Data acquisition and analysis were performed using Analyst software 2.6.1 (AB/SCIEX) and MultiQuant 3.0. Manual inspections on the data were also performed to rule out the false positive results, such as inconsistent baseline integration for peptide transitions, noise background and different retention times for analyte and corresponding IS peptide.

6.3.7 Data processing and protein quantification

The integrated peak areas for multiple transitions were averaged to represent the 'peak area' for one particular peptide (Kito and Ito, 2008). If two or more of the transitions from the same peptide failed to work, the peptide was filtered out (Zhang et al., 2011). Then the peak area of each unlabeled analyte peptide was compare to that of corresponding QconCAT peptide to generate a ratio (relative intensities). For one particular protein, the sum of intensities of its signature peptides was used to estimate the protein abundance (Carrillo et al., 2010). The

expression of CSEP0064 was normalized with the housekeeping proteins GAPDH (*B. graminis* f. sp. *hordei*) and GAPDH (*H. vulgare*), respectively.

6.4 Individual contributions

Mark Bennett (Imperial College London) performed the *in silico* analyses, MRM-MS and raw data acquisition.

I performed the plant material dissection, protein extraction, QconCAT expression and labelling, protein digestion and data processing for protein quantification.

6.5 Results

6.5.1 Isolation of haustoria

I used an enzymatic method to isolate *B. graminis* f. sp. *hordei* hasutoria from epidermal cells of infected barley. After microscopy of isolated haustoria that stained with WGA-Alexa Fluor 488, I observed that most of the isolated haustoria were highly branched and intact. Sometimes the separated haustorium was still surrounded by the plant-derived extrahaustorial membrane (Figure 6.4.a, arrow). Apart from haustoria, limited amount of plant debris, which were small enough to pass through the 70 μm nylon mesh sieve, were observed as well (Figure 6.4.b, red arrow).

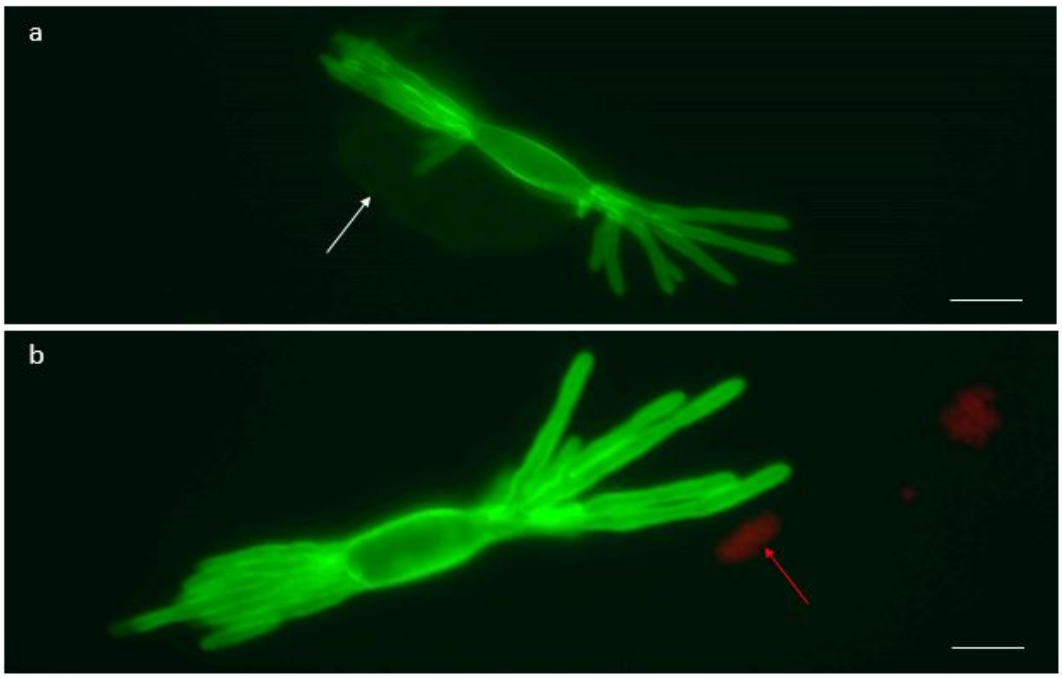

Figure 6.4 Isolated haustoria visualized by epifluorescence microscopy after staining with WGA-Alexa Fluor 488. Bar = 5 µm. (a) A multidigitated haustorium and the perihasutorial membrane (arrow). (b) The pelleted haustorial complex (from 5.3.1) consisted of not only haustoria, but also some plant chloroplasts (autofluoresent red, red arrow).

6.5.2 MRM-MS of QconCAT proteins

Prior to spiking the analytes with isotopically labelled QconCAT proteins, the total bacterial proteins, which contained IPTG-induced QconCAT, were examined by SDS-PAGE and MRM-MS. The expected size of synthetic QconCAT protein was 36.4 kDa. After Coomassie Blue staining, I observed an abundant band sized just above 35 kDa (Figure 6.5.a, arrow) in the induced bacterial proteins. There was no band distinguishable at the same position in non-induced control sample. Incomplete labelling of the QconCAT interferes with subsequent quantification of target proteins. I therefore investigated if there was unlabeled peptides by extracting and comparing heavy and light ions using Analyst software. There was no light peptide detected (Figure 6.5.b), meaning a 100% labelling efficiency of QconCAT proteins. Next, the existence and performance of each peptide were confirmed by the extracted-ion

chromatogram. All the peaks representing proteotypic peptides were sharp, and without obvious noise background (Figure 6.5.c). The number of peptides detected was consistent with the QconCAT design (Table 6.2).

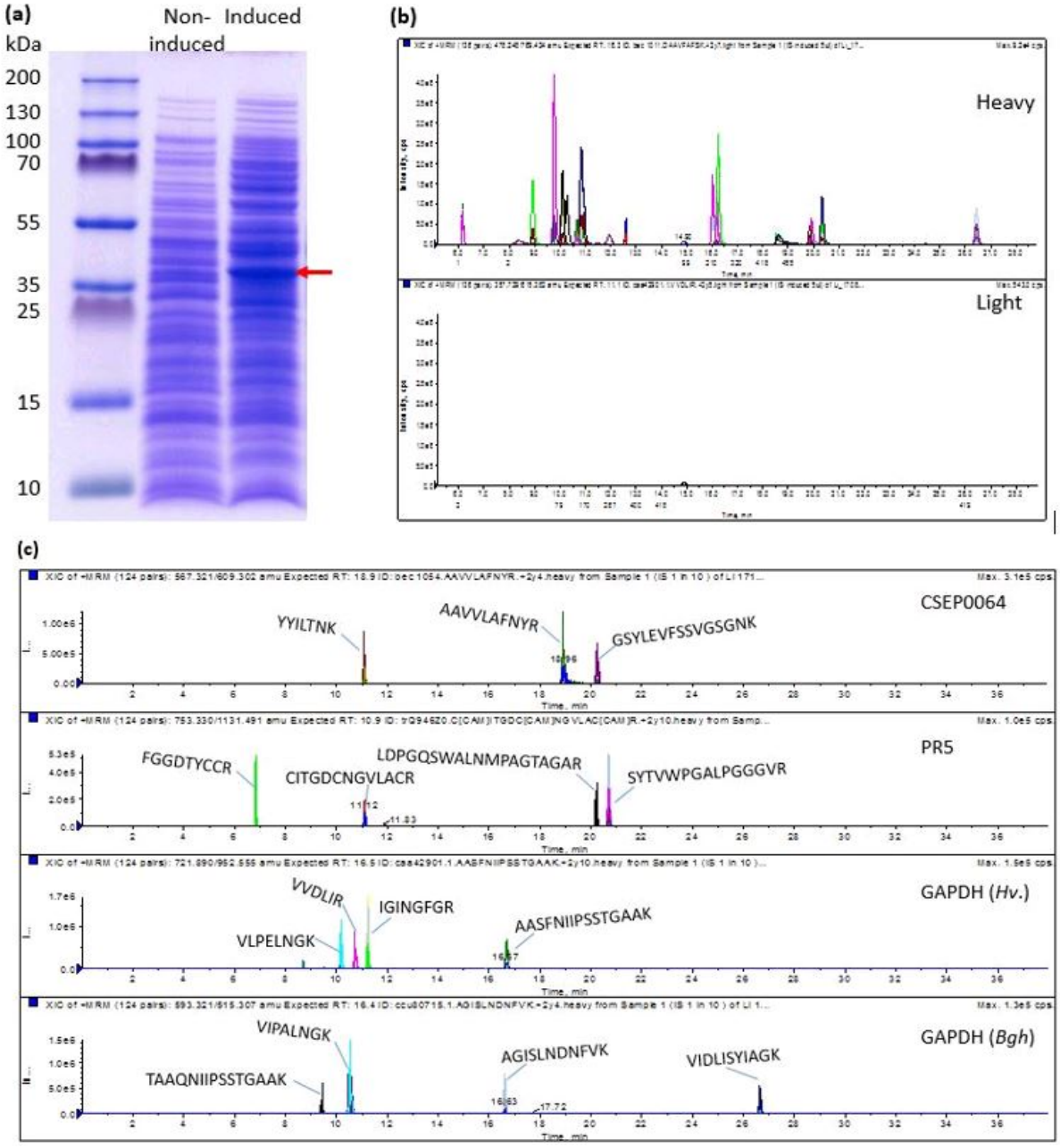

Figure 6.5 Validation of QconCAT protein by SDS-PAGE and MRM-MS. (a) The expression of synthetic QconCAT was induced by IPTG in *E.coli* BL21. Then total bacterial proteins from induced and non-induced samples were electrophoresed and stained with Coomassie Blue. An abundant band sized around 36 kDa (arrow) appeared in the induced sample,

representing the successful induction of QconCAT. (b) Extracted heavy and light ion chromatograms from MRM-MS analysis of induced bacterial proteins. There was no unlabeled peptides (the lower lane). (c) Chromatograms of target proteins that consisted of multiple proteotypic peptides. The peaks were labelled with corresponding peptides. As expected, all the peaks were outstanding without noise background.

6.5.3 Optimization of trypsin digestion for HA and ED-HA samples

The same amount of bacterial proteins (from cultures induced to produce the recombinant protein) were then spiked into the samples from four different dissected materials, and subjected to protein digestion and MRM-MS. None of the target peptides were detectable in HA and ED-HA samples (Figure 6.6.a). These two samples were prepared using a cellulase-based method, whereas EP and ED did not. I therefore tested whether the cellulase inhibited the trypsin digestion by analyzing the internal standard alone and internal standard mixed with cellulase. All target peptides were detected with clear peaks in the former sample (Figure 6.6.a), whereas only a few unspecific signals present in the latter sample (Figure 6.6.b).

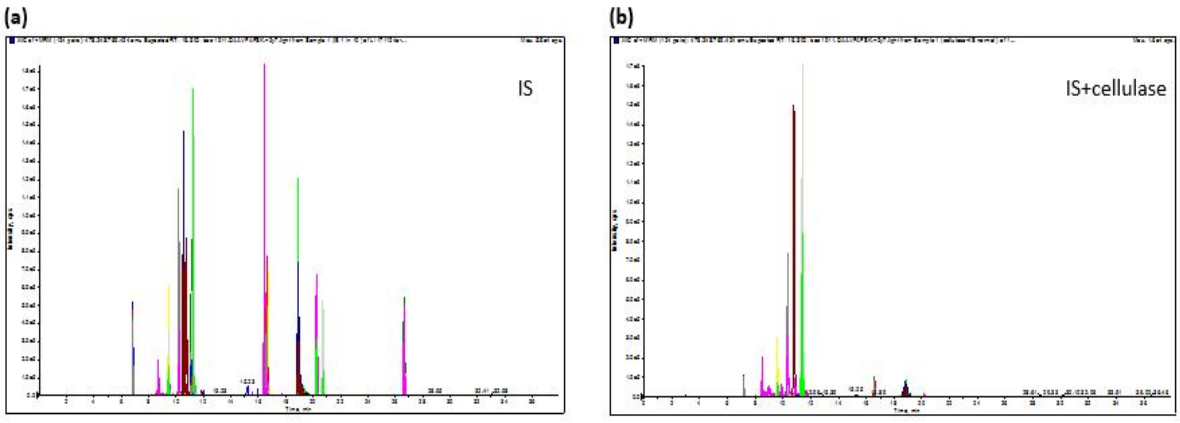

Figure 6.6 Cellulase inhibited the trypsin digestion. (a) When digesting the internal standard protein alone, many peaks were generated by MRM-MS. (b) When mixing the internal standard with cellulase, the digestion was inhibited. A few unspecific signals were observed.

Denaturation and reduction are two key steps before trypsin digestion, and incubation of proteins with DTT and higher concentration of urea at higher degree is usually suggested (Scheerlinck et al., 2015, Gundry et al., 2009). I then increased the urea concentration to 7M and the temperature of reduction step from 42 ℃ to 56 ℃. Using this method, multiple target peptides were detected in the samples containing cellulase, such as HA (Figure 6.7.b).

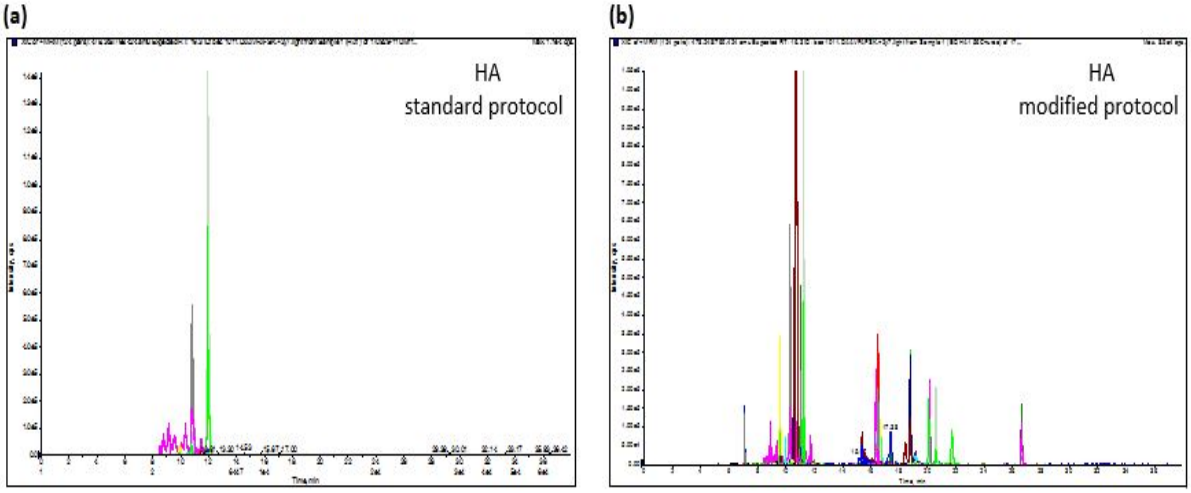

Figure 6.7 Chromatograms generated for HA samples that digested using standard protocol and modified protocol. (a) There was no target peptide detected in this sample while using the standard protocol. (b) When using the modified protocol to digest the same HA sample, many peaks that representing proteotypic peptides were generated through MRM-MS.

6.5.4 Detection and relative quantification of CSEP0064

6.5.4.1 Quantification of housekeeping proteins

Prior to the quantitative analysis of CSEP0064, the relative abundances (unlabeled/labelled) of two housekeeping proteins GAPDH (*B. graminis* f. sp. *hordei*) and GAPDH (*H. vulgare*) were calculated. These will then be used as reference standards against which the "specific quantity" of the CSEP is determined. As shown in Figure 6.8, the overall levels of two proteins varied among four dissected tissues. The fungal intracellular marker protein GAPDH was abundant in EP sample (Figure 6.8.a), which contains sporulating hyphae. The relative levels

of GAPDH (*B. graminis* f. sp. *hordei*) were significantly lower in the other two samples containing fungal material: ED and HA. In ED-HA sample that collected after removal of fungal haustoria, GAPDH (*B. graminis* f. sp. *hordei*) was barely detectable (value smaller than 0.1); this represents the contamination of fungal material in the ED-HA (plant) sample. In comparison with the fungal housekeeping protein, GAPDH (*H. vulgare*) was at very low level in EP and HA (Figure 6.8.b). As expected, the barley housekeeping protein was highly abundant in ED (relative abundance of 60). Note that ED-HA consisted of plant cytoplasm fractions and the added cellulase (used for haustoria isolation); here, the relative abundance of GAPDH (*H. vulgare*) was 0.4.

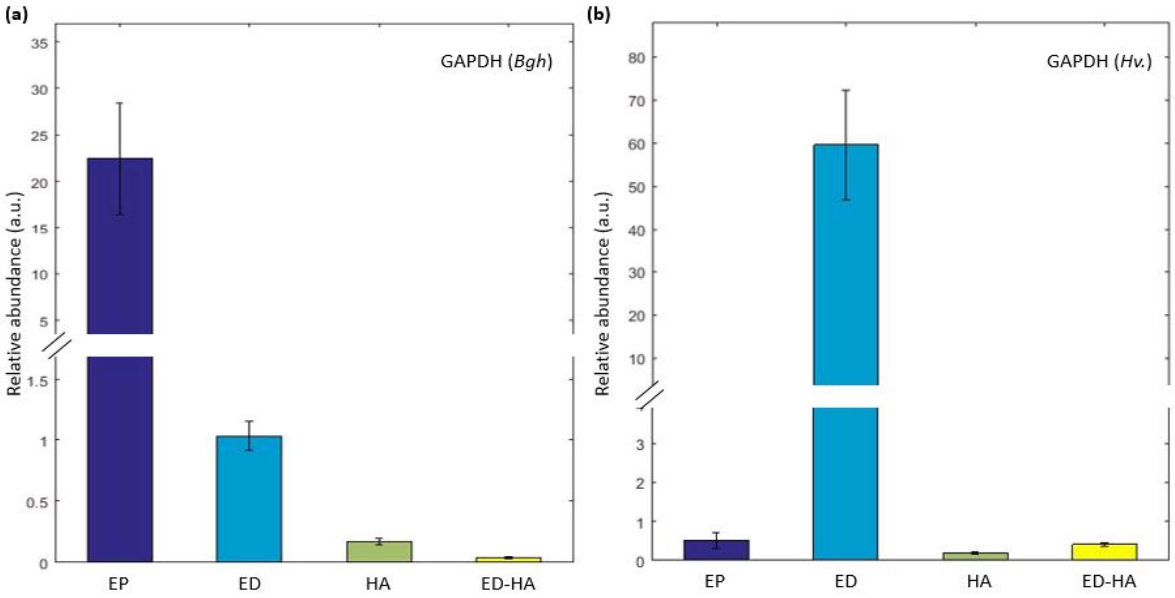

Figure 6.8 Relative abundances of housekeeping proteins GAPDH (*B. graminis f. sp. hordei*) and GAPDH (*H. vulgare*) in different dissected materials. The relative abundance for one particular protein was the ratio of its unlabelled form/labelled form. Roughly 80 infected leaves were used for dissection (every independent experiment), and then subjected to protein extraction. All digestions started with the same amount of total proteins (45 µg of analyte + 5 µg labelled internal standard). (a) The relative abundance of the fungal housekeeping protein in EP, ED, HA and ED-HA was 22.5, 1.0, 0.2 and 0.04, respectively. (b) Barley GAPDH was expressed in EP, ED, HA and ED-HA with relative values of 0.5, 59.5, 0.2 and 0.4, respectively. The error bars were calculated based on three independent biological replicates. *Bgh* for *B. graminis* f. sp. *hordei*, and *Hv.* for *H. vulgare*.

6.5.4.2 Relative quantification of CSEP0064

The performance of every single proteotypic peptide that constitute CSEP0064 was investigated first. Out of total three signature peptides, two of them were detectable and behaved consistently among four dissected tissues (Supplementary Figure 4). The other one (GSYLEYFSSVGSGNK) was not detectable in HA or ED-HA samples. Thus, the protein level of CSEP0064 was calculated based on two peptides: AAVVLAFNYR and GSYLEVFSSVGSGNK.

The labelled CSEP0064 standard was used to calculate the levels of unlabeled (native) CSEP0064, which was then further compared with two housekeeping proteins (Figure 6.9). First, it was quantified against the fungal GAPDH protein. In epiphytic (EP) samples, CSEP0064 was less than one percent of GAPDH (*B. graminis* f. sp. *hordei*) (too low to display in Figure 6.9.a); in contrast, it was nearly nine times higher than that of the fungal housekeeping protein in proteins from infected epidermis (ED) (Figure 6.9.a). The highest proportion of CSEP0064 to GAPDH (*B. graminis* f. sp. *hordei*) was found in plant epidermal cytoplasm fraction (ED-HA) (ratio of 41). In haustoria (HA) samples, CSEP0064 was 17-fold higher than the fungal housekeeping protein. I next calculated the abundance of CSEP0064 relative to the barley endogenous protein GAPDH (Figure 6.9.b). In EP and HA samples, which mainly consisted of fungal structures, the ratios of CSEP0064/ GAPDH (*H. vulgare*) were 3.6 and 15.5, respectively. Strikingly, while CSEP0064 and barley housekeeping protein were nearly at the same level (ratio of 0.9) in ED, the level of CSEP0064 in ED-HA was more than three times (ratio of 4.3) higher than the plant housekeeping protein.

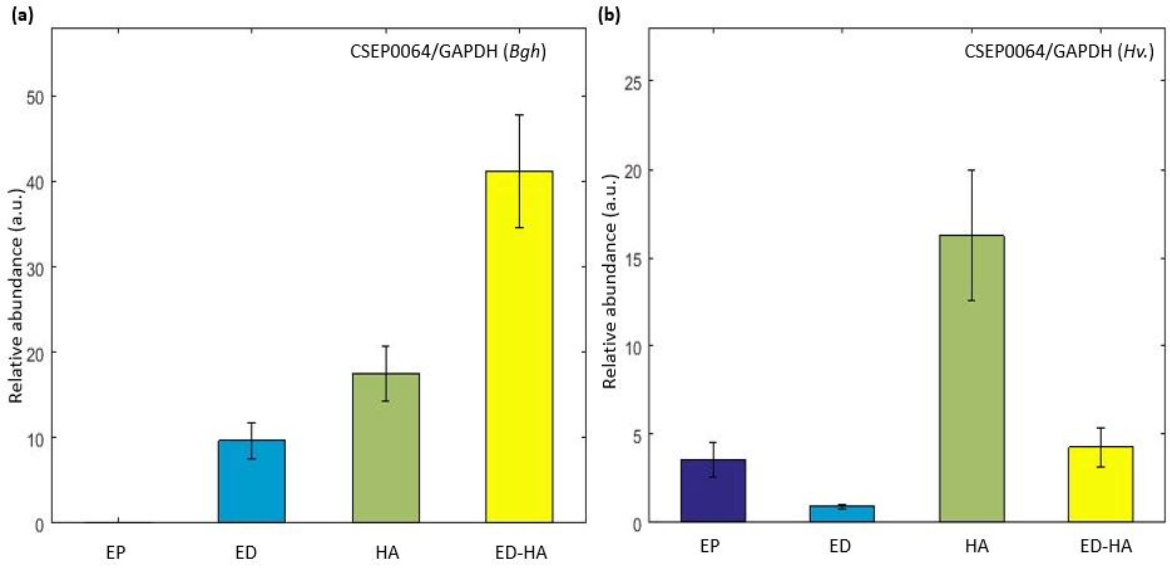

Figure 6.9 Relative level of CSEP0064 to two housekeeping proteins. (a) CSEP0064 was more abundant (> 10 times) than in ED, HA and ED-HA samples. The ratio of CSEP0064/GAPDH (*B. graminis* f. sp. *hordei*) was too small to display. (b) CSEP0064 showed higher abundance than in EP, HA and ED-HA, whereas displayed slightly lower level than GAPDH (*H. vulgare*) in ED.

6.6 Discussion

Accurate identification and quantification of proteins from highly complex samples is a main challenge for proteomic studies (Maiolica et al., 2012, Wiśniewski and Mann, 2016). This is particular true when investigating biotrophic pathogens, such as *B. graminis* f. sp. *hordei*. As shown in chapter 1 (Figure 1.1), *B. graminis* f. sp. *hordei* develops an intimate relationship with barley by establishing the feeding structure haustoria inside plant epidermal cells (Both et al., 2005, McGrann et al., 2014). Thus, the fungal structures are present both inside and outside plants. In this study, I dissected the infected barley leaves to obtain four representative structures: epiphytic mycelia embedded in cellulose acetate membrane (EP), epidermis containing haustoria (ED). The later tissue was further fractionated to obtain isolated haustoria (HA) and lysate containing cytoplasm from epidermal cells minus the haustoria (ED-HA).

Although the dissection of sporulating hyphae and epidermis has been reported widely when studying *B. graminis* f. sp. *hordei* effectors (Bindschedler et al., 2009, Bindschedler et al., 2011, Amselem et al., 2015b), isolation of *B. graminis* f. sp. *hordei* haustoria has been reported only once (Godfrey et al., 2009). In their study, the haustoria were isolated using a filtration and gradient centrifugation-based method, which is relatively laborious. In contrast, I used a simpler way to isolate haustoria from infected barley leaves. The release of haustoria was achieved by enzymatically degrading the epidermal cell walls. Importantly, this method enabled the collection of plant cytoplasm fractions, which may help in investigating the secretion of effectors.

In this study, I used MRM-MS to identify and quantify CSEP0064 in different tissues. The quantification of CSEP0064 was extensively based the concept of 'relative ratio' by taking advantage of isotopically-labelled QconCAT protein and housekeeping proteins. Usually, housekeeping proteins are involved in basic cell maintenance. Thus, we assume them to be stably expressed and present constantly in different cell types (Ludwig et al., 2012, Zauber et al., 2013, Hu et al., 2016).

GAPDH is an enzyme which functions in glycolysis (Barber et al., 2005, Kozera and Rapacz, 2013). The GAPDH protein, one the most widely used housekeeping proteins, was selected for normalization in this study because of its good performance in our previous qPCR experiments (Pennington et al., 2015); moreover, it has been identified in *B. graminis* f. sp. *hordei* haustoria in previous shotgun proteomics (Godfrey et al., 2009). GAPDH (*B. graminis* f. sp. *hordei*) was at the highest levels in EP. This is expected as this sample contained the highest fungal biomass. The detection of GAPDH (*B. graminis* f. sp. *hordei*) in ED-HA is likely to indicate the contamination from lysed fungal cells/haustoria. As expected, the plant GAPDH (*H. vulgare*) was highly abundant in ED. As I used the same quantity of total proteins from each dissected samples for MRM-MS, and ED-HA sample contained considerable amount of cellulase, it is

not surprising that GAPDH (*H. vulgare*) level was low in ED-HA. Although the barley housekeeping protein was also detectable in EP and HA, this was probably due to the contamination of plant proteins in these fractions. For example, when I peeled the cellulose acetate membranes from barley, the leaf hairs could also be removed. In the HA sample, the haustorial pellet contained plant debris and plant-derived extrahaustorial membranes, as was evident when the material was observed under the microscope (Figure 6.4). The detection of housekeeping proteins from contaminations highlights the high sensitivity of my MRM-MS system.

It should be noted that although we assume housekeeping protein levels to be constant, it could be they are not, even in closely related samples For example GAPDH (*B. graminis* f. sp. *hordei*) was highly abundant in EP, whereas was just at a detectable level in HA. Given that this was the first MRM-MS study of *B. graminis* f. sp. *hordei,* there was limited prior-information of the selection of reference proteins. With the increasing number of studies that showing the instability of housekeeping proteins (Hu et al., 2016, Zauber et al., 2013), using a combination of several housekeepers may become necessary rather than optional (Wiśniewski and Mann, 2016) and this could be applied to future *B. graminis* f. sp. *hordei* proteomic studies as well.

Regardless of the varying levels of two housekeeping proteins in different fractions, we used them as a reference for *B. graminis* f. sp. *hordei* and barley proteins. Relative to GAPDH (*B. graminis* f. sp. *hordei*), the level of CSEP0064 was dramatically higher in ED than in EP: the data suggests an approximately 103-fold increase in the accumulation of CSEP0064 protein in infected cells, assuming a constant level of GAPDH (*B. graminis* f. sp. *hordei*) in the two tissues (ED and EP). This is consistent with the induction of CSEP0064 gene expression previously reported during the early infection development (Pennington et al., 2015) and with the relatively high proportion of CSEP0064 mRNA in haustoria vs epiphytic hyphae

(Pennington et al., 2015, Spanu et al., 2010, Pedersen et al., 2012). It is very important to note that CSEP0064 was about four-fold higher in ED-HA than in HA. This indicated that this effector was secreted by the haustoria, as is expected for a protein whose gene encodes a classical secretion peptide (Pedersen et al., 2012). When using GAPDH (*H. vulgare*) as a normalizer, CSEP0064 was at a significant higher level than the barley housekeeping protein in EP and HA. This was expected because fungal materials account for the vast majority of these two samples. Although the secretion of CSEP0064 by haustoria has been predicted through proteomic and genomic studies (Bindschedler et al., 2011, Pedersen et al., 2012), my results, for the first time, clearly showed it at a peptide level using quantitative data.

It is worth noting that CSEP0064 was more than four times as abundant as the GAPDH (*H. vulgare*) protein in the ED-HA sample, which represented the plant cytoplasm fractions. This suggest that not only the CSEP0064 is secreted by the haustoria, but also that the effector appears to be taken up by the cellular fraction containing GAPDH (*H. vulgare*), i.e. the plant cytoplasm. This is exceptionally important. There is much indirect evidence that powdery mildew effector proteins are ultimately delivered to the host cytoplasm (Koller et al., 2018, Jin et al., 2018). For example, Avr effectors related to CSEP0064 are recognised by host R proteins localised in the cytoplasm (Praz et al., 2017, Bourras et al., 2015). To my knowledge, this is the first time that direct biochemical evidence that is consistent with the uptake of CSEPs by the host plant.

MRM-MS has the potential for detection and quantification of post-translational modifications (Mirzaei et al., 2008, Brownridge et al., 2011). In that case, synthetic signature peptides must carry the known specific amino acid residues of post-translational modifications (Pan et al., 2009, Liu et al., 2013). For example, if the quantification of phosphorylation is needed, reference peptides are synthesized to mimic the natural unmodified peptides and modified peptides with a target phosphorylation site and then used as internal standards

(Kettenbach et al., 2011). Accordingly, the QconCAT strategy I used in this study would not be suitable for the detection of modified peptides. In other words, constant detection of two signature peptides of CSEP0064 suggests that they did not contain post-translational modification sites. Conversely, the third peptide escaped detection in HA and ED-HA could due to the post-translational modification site happening there. It is also worth noting that, MRM-MS possess the capability of monitoring large number of target proteins by design and using multiple synthetic internal standards (Lawless et al., 2016). Although only one effector protein was investigated here, the study presents here suggests a potential way to study multiple low-abundance CSEPs simultaneously.

6.7 Conclusions

In the present study, I used the MRM-MS method and the QconCAT strategy to detect and quantify *B. graminis* f. sp. *hordei* effector CSEP0064 in infected host leaves successfully. Dissection of barley leaves including a newly-developed enzymatic way for haustoria isolation, enabled the detection of CSEP0064 in different *B. graminis* f. sp. *hordei* lifecycle-related structures. CSEP0064 was highly expressed in HA and ED-HA tissues, suggesting it was secreted by the haustoria and delivered into plant cell.

7 General discussion and conclusions

Recent advances in 'omics' technologies such as proteomics, genomics and transcriptomics strikingly accelerated our understanding of the obligate biotrophic barley powdery mildew fungus (Bindschedler et al., 2016). The discovery of genes up-regulated specifically during infection (Both et al., 2005b, Both et al., 2005a); identification of proteins expressed exclusively in haustoria (Bindschedler et al., 2009, Bindschedler et al., 2011); more importantly, the sequencing of several powdery mildew species including *B. graminis* f. sp. *hordei* (Spanu et al., 2010), led to the identification of hundred CSEPs (overlap with BECs with five exceptions) (Pedersen et al., 2012). Following this, many efforts have been made to characterize the activities of these effector proteins. For example, host-induced gene silencing was used to identify whether they play a role in the fungal virulence (Pliego et al., 2013, Ahmed et al., 2016, Whigham et al., 2015, Aguilar et al., 2015). These studies suggest that some CSEPs are probably dispensable for pathogen virulence, while others such as CSEP0064 and CSEP0264 are essential for barley powdery mildew infection (Bourras et al., 2018, Bourras et al., 2016). Moreover, yeast two-hybrid assay was used to identify the putative host interactors of different CSEPs including CSEP0064 (Ahmed et al., 2015, Pennington et al., 2016). However, the function of their interactions is still unclear. In summary, our knowledge for the mode of action of CSEPs is still scarce.

Prior to the beginning of my PhD, the *P. fluorescens*-based modified T3SS EtHAn was developed and proven to be capable of delivering foreign effectors into wheat leaves (Thomas et al., 2009, Upadhyaya et al., 2014). In addition, a wheat mapping population WAGTAIL that contains more than 400 wheat varieties was developed. Taken together, these two provided the possibility for delivering *B. graminis* f. sp. *hordei* effectors into non-host wheat plants, and screen for effector-specific responses. Moreover, the anti-CSEP0064 polyclonal primary

antibody was raised in rabbits, which provided the possibility of determining the expression of CSEP0064 in infected host plant. Taking advantage of these materials, I was able to carry out the investigations of CSEP0064 and CSEP0264 in non-host plant wheat and host plant barley.

Previously, our knowledge of NHR against *B. graminis* f. sp. *hordei* was mostly collected from the interactions between this pathogen and dicots (Lipka et al., 2008, Collins et al., 2003, Hückelhoven et al., 2000). My study successfully delivered two CSEPs into non-host monocot plant, wheat, using the modified T3SS EtHAn. Although the non-pathogenic bacterium *P. fluorescens* triggered PTI on 125 lines (out of total 441), which posed a challenge for screening for effector-triggered responses. I developed an image processing pipeline to quantify the response levels using MATLAB. Finally, CSEP0064 was found to enhance EtHAn-triggered PTI on Rialto and Abbot, and CSEP0264 enhanced the PTI on Rialto and Madrigal.

To further define if the observed effector-triggered response is a consequence of ETI, it is then tested if the recognition of CSEPs triggered the susceptibility change of wheat, as ETI is considered to be associated with SAR (Henry et al., 2013, Truman et al., 2007).

SAR is a phenomenon whereby prior (primary) infection or treatment renders uninfected systemic (distal) tissues more resistant against subsequent infections (Fu and Dong, 2013, Conrath, 2006). Moreover, prior infection can trigger enhanced systemic susceptibility as well (Torres et al., 2017, Ji et al., 2016). However, there has been few studies described the SAR or SAR-like phenomenon in monocots (Peng et al., 2011, Yang et al., 2013), while no previous report demonstrated induced systemic susceptibility in monocots. Using the EtHAn-infiltration as a potential 'inducer', I tested the susceptibility change in wheat, both locally and systemically. Importantly, EtHAn-infiltration with CSEPs either triggered systemic resistance or induced systemic susceptibility to subsequent challenge with *B. graminis* f. sp. *tritici* in three lines Rialto, Abbot and Madrigal, which responded to the fungal effectors during screening.

On the other two control lines Cerco and Hardi that did not respond to effectors, there was no susceptibility change observed at systemic level. The results suggest that the effector-specific responses I observed during screening is a visible symptom of ETI. This means there are genes in wheat that encode the recognition and differentiation of barley powdery mildew effectors. Moreover, the recognition of CSEPs did not alter the systemic wheat immunity against non-adapted pathogen *B. graminis* f. sp. *hordei*. This suggests that wheat non-host resistance and host resistance are controlled by different systemic signalling pathways or basal plant immunity is sufficient to restrict non-host pathogen invasion and additional up-regulation of systemic immunity does not provide any additional advantage.

Quantitative PCR (qPCR) has been used to determine the expression patterns of CSEPs in different studies (Whigham et al., 2015, Pliego et al., 2013, Pennington et al., 2015). However, the expression of CSEP0064 at protein level rather than mRNA level, has not been clearly elucidated. Using western blots, I detected the *B. graminis* f. sp. *hordei* effector CSEP0064 in infected barley leaves and isolated haustoria. Additionally, a protein complex shown as a diffuse signal around 200 kDa was observed in all fungal structure-containing materials. This species was termed 'Big CSEP' and may be a glycosylated form of CSEP0064, as it could be completely degraded by TFMS. MRM-MS has the potential to detect and quantify very low-abundance proteins in complex samples (Maiolica et al., 2012, Liu et al., 2013). My study, for the first time, utilized MRM-MS to quantify the level of *B. graminis* f. sp. *hordei* effector in different fractions of infected barley leaves, using GAPDH (*B. graminis* f. sp. *hordei*) and GAPDH (*H. vulgare*) as the reference for fungal and plant proteins. CSEP0064 is widely assumed to be secreted by the haustoria and ultimately delivered into the plant cytoplasm (Pliego et al., 2013, Bindschedler et al., 2009, Bindschedler et al., 2011, Praz et al., 2017), my quantitative results from MRM-MS provide direct evidence for the secretion and uptake of CSEPs by host plant cells. Additionally, one target peptide of CSEP0064 was not detectable in

HA and ED-HA by MRM-MS. Based on the western blot results, CSEP0064 is likely to be *O*-glycosylated. It is possible that the peptide escaped detection is due to the post-translational modification happening there.

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I'm deeply grateful to my parents and my brother. Thank you for the financial support that makes me live comfortably all these years. Thank you for the emotional support that makes me live as what I like to. Special thanks to my bother for all your WeChat red packages.

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Additional outcomes of this Ph.D.

- Pennington, H. G., Linhan, L. & Spanu, P. D. (2015). Identification and selection of normalisation controls for quantitative transcript analysis in *Blumeria graminis*. Molecular Plant Pathology. 1(1),1-9
- Linhan, L., Vincent S., Kung A. & Spanu, P.D. EtHAn-delivered CSEPs trigger systemic changes in susceptibility to powdery mildew, in wheat (in preparation)
- Linhan, L., Mark, H. Bennett., Collier B.,& Spanu, P.D. Detection and quantification of CSEP0064 using MRM-MS (in preparation)

Supplementary

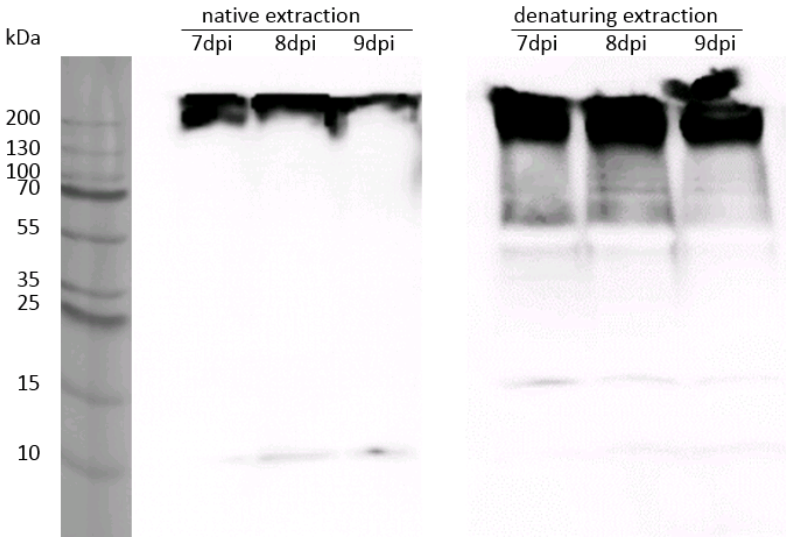

Figure 1. Western blot of proteins extracted with different lysis buffers. Total proteins of heavily infected (7 dpi, 8 dpi and 9 dpi) barley leaves were extracted with native lysis buffer (a) and denaturing lysis buffer (b), respectively. Same amount of proteins (30 µg) were then separated using Tris-Glycine system, and then subjected to western blot with anti-CSEP0064 antibody. 'Big BEC' was observed in all of those samples.

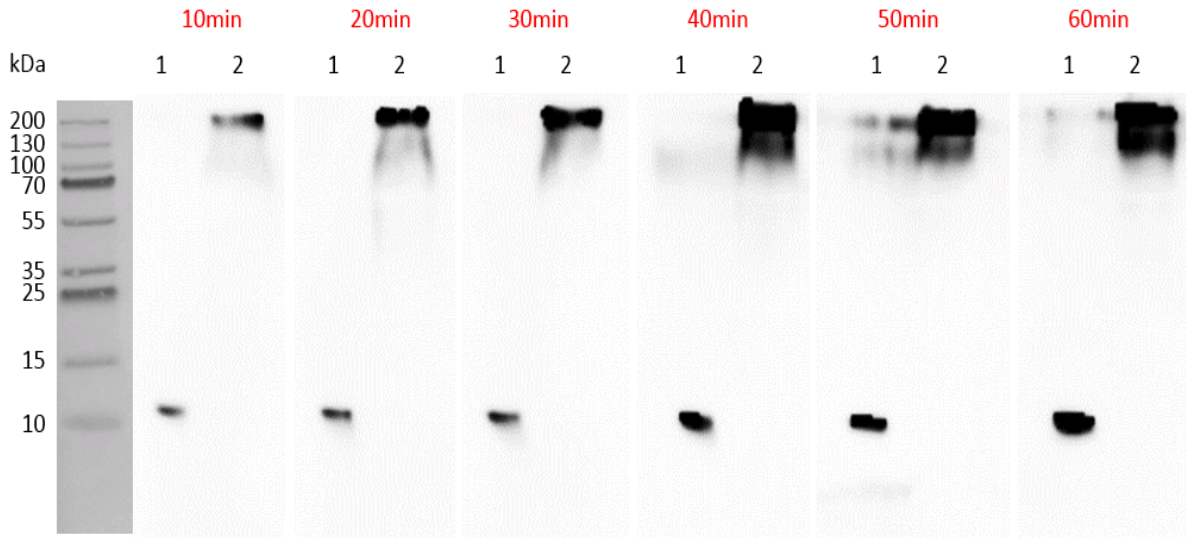

Figure 2. Western blot with different transfer time. Recombinant CSEP0064 (left) and 9 dpi barley (right) were used to show the effects of transfer time on signal intensity. All protein loadings for each transfer time are identical. Both signals for recombinant CSEP0064 and for 'Big BEC' increased as the transfer time increasing.

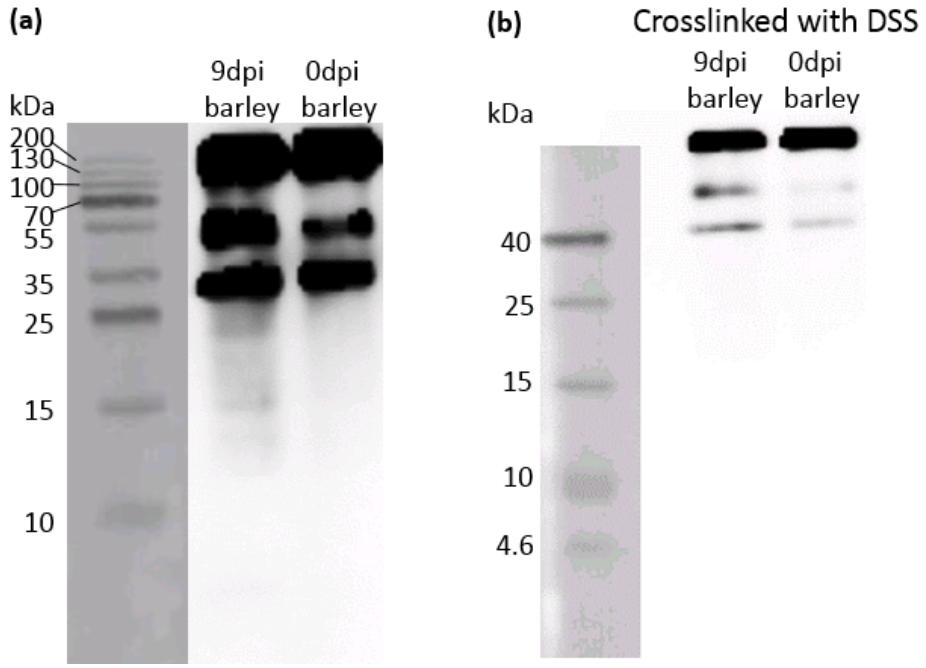

Figure 3. Western blots of immunoprecipitated samples (+/- crosslinking with DSS). Total proteins from 9 dpi barley were immunoprecipitated with anti-CSEP0064 antibody and then immunoblotted with anti-CSEP0064 primary antibody. 0 dpi sample was included as a negative control. (a) Standard IP without crosslinking. Multiple intensive bands from co-eluted antibody

were detected. The highest even masked the area around 200 kDa. (b) Improved IP with crosslinking. The signal from co-eluted antibody was largely reduced, but there was still an intensive band signal at the top. There was no band detected at the expected size of CSEP0064, indicating the low efficiency/failure in precipitating CSEP0064.

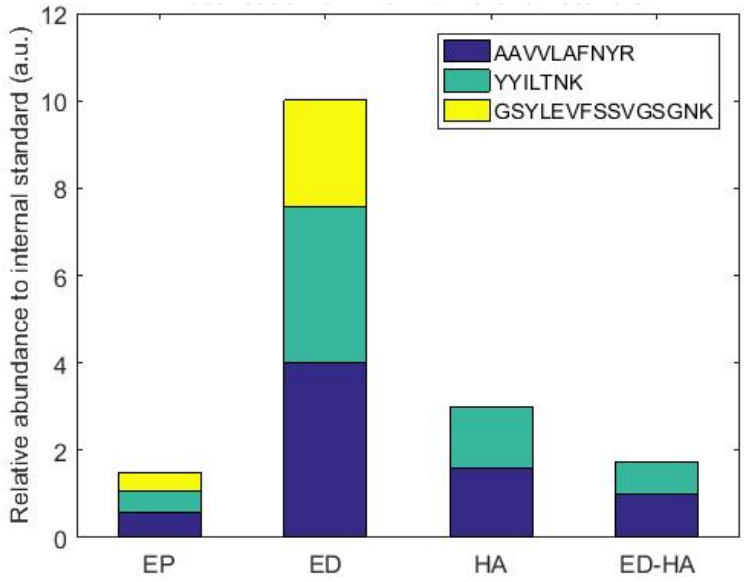

Figure 4. Relative abundance of three peptides of CSEP0064. Peptide GSYLEVFSSVGSGNK was not detectable in HA and ED-HA samples.

Table 1. Preparation of amino acid stocks based on different solubility.

Groups	Solubility	Amino acids
1	H_2O	alanine, asparagine, glutamate, glutamine, glycine, methionine, proline, serine, threonine, and valine
2	HCl	arginine, histidine, cysteine and lysine
3	0.1 % PEG in H_2O	isoleucine, leucine and phenylalanine
4	NaOH	aspartate, tryptophan and tyrosine

Recipe for 1000x trace element solution in total 10 ml: EDTA (0.5 g), $FeCl_3.6H_2O$ (0.083 g), $ZnCl_2$ (8.4 mg), $CoSO_4.2H_2O$ (1.3 mg), H_3BO_3 (1 mg), $M_nCl_2.6H_2O$ (0.16 mg) and $Na_2MoO_4.2H_2O$ (1 mg)

Reference

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