# STUDY ON THE MODE OF ACTION OF CEP PEPTIDE AS AN ENDOGENOUS SUPPRESSOR IN *ARABIDOPSIS THALIANA*

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**APRILIA NUR FITRIANTI**

**Graduate School of Environmental and Life Science**

**(Doctor Course)**

**OKAYAMA UNIVERSITY**

# Study on the mode of action of CEP peptide as an endogenous suppressor in *Arabidopsis thaliana*

シロイヌナズナの内生サプレッサー
としての CEP ペプチドの作用機序に関する研究

**Aprilia Nur Fitrianti**

Graduate School of Environmental and Life Science
Division of Agricultural and Life Science
Okayama University, Japan
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Written under supervision of Professor Kazuhiro Toyoda and co-supervised by Professor Yuki Ichinose and Associate Professor Yoshiteru Noutoshi.

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# Summary

An endogenous suppressor (ES) is defined as constitute(s) synthesized by plant, which suppress(es) elicitor-induced defense responses when applied to the plant (Nasu et al. 1992). The role of ES is considered to prevent excessive defenses and fine-tune the trade-off between the growth and the defense. Recently, Arabidopsis ES was shown not only to suppress PAMP-induced defenses, but also to induce susceptibility to non-adapted pathogens (Mai et al. 2020). Further LC-MS/MS analysis of the Arabidopsis ES identified a peptidic fragment corresponding to a c-terminally encoded peptide 5 (CEP5). In Arabidopsis, the CEP peptide-encoding genes comprise a large family (*CEP1* to *CEP15*), with individual members encoding proproteins containing one to five CEP domains at or near the C-terminus, yielding small peptides consisting of 15 amino acids through post-translational modifications. The resulting CEP peptides are usually hydroxylated on the proline residues through a putative hydroxylase. In this thesis, using the synthesized CEP5p$^{hyp}$ (DFR{HYP}TT{HYP} GHS{HYP}GIGH; CEP5p$^{hyp}$), I examined its activity for defense suppression and also discussed the role in plant immunity.

In chapter II, I started to investigate the effect of CEP5p$^{hyp}$ on Arabidopsis immunity, especially focusing on its induction of disease susceptibility to non-adapted fungal and bacterial pathogens. Similar to the Arabidopsis ES, CEP5p$^{hyp}$ allowed non-adapted pathogens to cause symptoms, when applied exogenously to Arabidopsis Col-0. *Colletotrichum tropicale* (*Ctro*) or *Mycosphaerella pinodes* (*Mp*), which are pathogenic to mulberry or pea, respectively, did not cause disease symptom on the Col-0 leaves, while symptoms were developed when each conidia were inoculated simultaneously with the CEP5p$^{hyp}$. Notably, on the Col-0 leaves with CEP5p$^{hyp}$, the conidia germinated to form hyphal-like structures, which directly penetrate epidermis without forming melanized appressoria. This is reminiscent of the hyphal tip-based entry (HTE) reported by Hiruma *et al*. (2010), who showed that *Ctro* employs the HTE on Arabidopsis *pen2* mutant. Similarly, induced susceptibility caused by CEP5p$^{hyp}$ was observed when challenged with a non-adapted bacterial pathogen, *Pseudomonas sryingae* pv. *tabaci* 6605 (*Pta* 6605), a causal agent of the wildfire disease on tobacco.

CEP peptide(s) is well characterized as a root-to-shoot signal molecule that is generated in roots that sense nitrogen (N) deficiency and subsequently compensates for N acquisition through interaction with typical LRR-type receptor kinases CEPR1/XIP1 and CEPR2 in the shoot vascular tissues. I also examined whether the CEPR1 and 2 are involved in the CEP5p$^{hyp}$-mediated defense suppression. The *cepr1-1*, *cepr2-1* and *cepr1,2* double mutant plants (No-0 background) were tested

for induction of susceptibility using *Ctro* or *Mp* as the non-adapted fungal pathogen. Interestingly, the CEP5p$^{hyp}$-mediated susceptibility was observed on the No-0 and *cepr* mutants when inoculated with *Ctro* or *Mp*. An additional *cepr1-3* with a different mutation allele in the Col-0 background also showed the same phenotype. On the basis of these results, it is likely that the signaling pathway underlying the CEP5p$^{hyp}$-caused defense suppression is mediated independently of CEPR1 and CEPR2, which are known to act on systemic signaling during N deficiency. Rather, an unidentified receptor(s) other than CEPR1 and CEPR2 may play a role the CEP5p$^{hyp}$-mediated disease susceptibility.

In chapter III, I further investigated whether the CEP5p$^{hyp}$-mediated susceptibility is effective against other bacterial pathogens. The use of model pathogenic bacteria of *P. syringae* pv. *tomato* DC3000 (DC3000) and its derivatives revealed that the Col-0 plants with CEP5p$^{hyp}$ also exhibited enhanced disease susceptibility to an avirulent *P. syringae* pv. *tomato* DC3000 *hrcC*$^{-}$ (*hrcC*$^{-}$) as well as one expressing *avrRpm1* (*avrRpm1*). Taken together, these observations clearly indicate that exogenous CEP5p$^{hyp}$ can suppress the Arabidopsis immunity via affecting the common pathway(s) or process(es) leading to the PTI and ETI. Subsequently, I tried to examine whether the CEP5p$^{hyp}$ suppresses or delays the Arabidopsis immune responses, by measuring the production of reactive oxygen species (ROS), activation of MAP kinases and the subsequent expression of defense-related genes as indicators of plant immunity. Adding CEP5p$^{hyp}$ to Arabidopsis Col-0 caused a suppression or delay both in flg22-induced ROS generation and MAP kinase activation, eventually resulting in insufficient expression of defense-related genes. Indeed, the concomitant presence of flg22 with CEP5p$^{hyp}$ clearly reduced the callose accumulation, a typical marker of PTI. In addition, exogenous CEP5p$^{hyp}$ reduced camalexin accumulation in Col-0 leaves exposed to silver nitrate as an abiotic elicitor. It is thus considered again that CEP5p$^{hyp}$ can modulate Arabidopsis immunity by targeting the common defense signaling pathway.

Finally, to understand the role of CEP peptides in modulating the Arabidopsis immunity, expression for *CEP* genes (*CEP1* to *CEP15*) were analyzed with Col-0 leaves challenged with DC3000, *hrcC*$^{-}$, *Pta* 6605 or *avrRpm1*, respectively. Except with the virulent DC3000, the *CEP1*, *CEP3*, *CEP5*, *CEP8* and *CEP9* genes were upregulated at 24 h after inoculation, some of which were shown to be induced in response to flg22 or salicylic acid (SA). These results support the hypothesis that CEP peptides prevent excessive defenses and fine-tune the trade-off between the growth and the defense. So far, studies on the activation of plant immunity, especially focusing on endogenous SA, have been extensively carried out, but little is known about the endogenous molecule(s) that

negatively control plant immunity. In recent year, in *Medicago truncatula* under high nitrogen condition, a certain CEP peptide, whose is induced in response to symbiotic association or Nod factor-treatment, is shown to regulate locally or systemically secondary infection of rhizobia via affecting ethylene signaling pathway (Zhu et al. 2020). It is thus now becoming clear that CEP peptides regulate not only plant growth, but also their association with microorganisms. In this scenario, individual CEP peptides from a huge gene family (yielding at least 24 peptides in Arabidopsis), likely play diverse roles i.e., growth, symbiotic association, and immunity. This study is thus expected to be a breakthrough in the field of plant-microbe interactions to open a new perspective on the regulatory mechanism of plant immunity.

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# Abbreviations

| | |
|---|---|
| ANOVA | analysis of variance |
| Avr | avirulence |
| cDNA | complementary DNA |
| C-terminus | carboxy-terminus |
| *C. tropicale / Ctro* | *Colletotrichum tropicale* |
| cfu | colony forming unit |
| dpi | day(s) post infection |
| ETI | Effector-triggered immunity |
| Fig. | Figure |
| h | hour(s) |
| hpi | hour(s) post infection/inoculation |
| hpt | hour(s) post treatment |
| HRP | horseradish peroxidase |
| KB | King's B |
| MAPK | mitogen-activated protein kinase |
| µM | micromolar |
| min | minute(s) |
| mM | millimolar |
| *M. pinodes / Mp* | *Mycosphaerella pinodes* |
| OD | optical density |
| PAMPs | pathogen-associate molecular patterns |
| PRRs | Pattern recognition receptors |
| PTI | PAMPs-triggered immunity |
| pv. | pathovar |
| qRT-PCR | quantitative reverse transcription polymerase chain reaction |
| RLCK | receptor-like cytoplasmic kinase |
| ROS | reactive oxygen species |
| RNA | ribonucleic acid |
| rpm | round per minute |

| | |
|---|---|
| RPM | resistance to *Pseudomonas syringae* pv. *maculicola* |
| sec | second(s) |
| SDS | sodium dodecyl sulphate |
| T3SS | type III secretion system |
| v/v | volume by volume |
| w/v | weight by volume |

# Chapter 1: General Introduction

## 1.1 The plant immune system

The first encounters between plants and pathogens commonly happened on the plant cuticle and cell wall. Therefore, the cuticle and cell wall act as the first physical barrier between plants and pathogens (Lee et al. 2017). The cuticle is present on the external surface of the aerial parts of the epidermal cell wall and mainly composed with cutin and on younger parts of plants, waxes (Agrios 2005; Bigeard et al. 2015). The cuticle plays an essential role as physical barriers against phytopathogens, although most of the fungal pathogens can penetrate the plants through mechanical force or secretion of cutinases to breakdown the cutin (Agrios 2005; Bigeard et al. 2015; **Fig. 1.1**). On the other hand, the plant cell walls comprise a hydrostatic polysaccharide-based skeleton which consists of cellulose microfibrils, hemicelluloses, pectin, and in woody or some other plants, lignin (Agrios 2005; Lampugnani et al. 2018). Besides, the plant cell walls provide structural support and together with cuticle, allow protection against biotic and abiotic stresses (Bigeard et al. 2015).

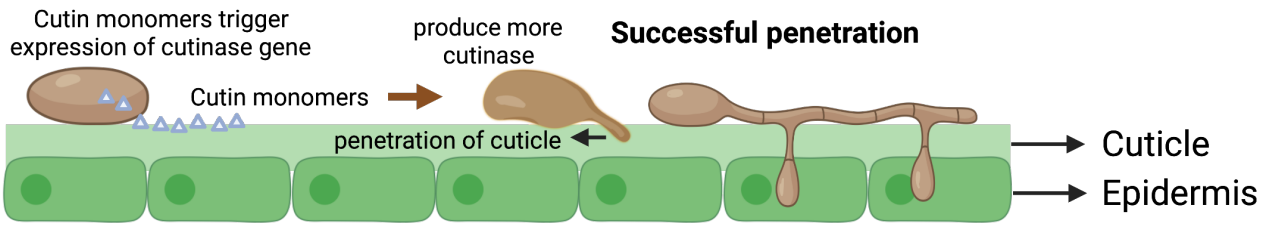



**Fig. 1.1 The illustrative representation of cuticle penetration by a germinating fungus spore.** The involvement of cutinase during the penetration process of the host plant cuticle by pathogenic fungi triggers expression of cutinase gene and induces the fungus to produce more cutinase, which allows successful penetration. Figure adapted from Agrios (2005), created with BioRender.com.

The pathogens that can break through the plants first physical barriers must deal with plants dynamic immune system. Plants can sense pathogens invasion by detecting conserved features that essential for pathogens whether for living or survival known as pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs) (Nicaise et al. 2009; Stam et al. 2014).

### 1.1.1 Pathogen-associate molecular patterns (PAMPs)-triggered immunity (PTI)

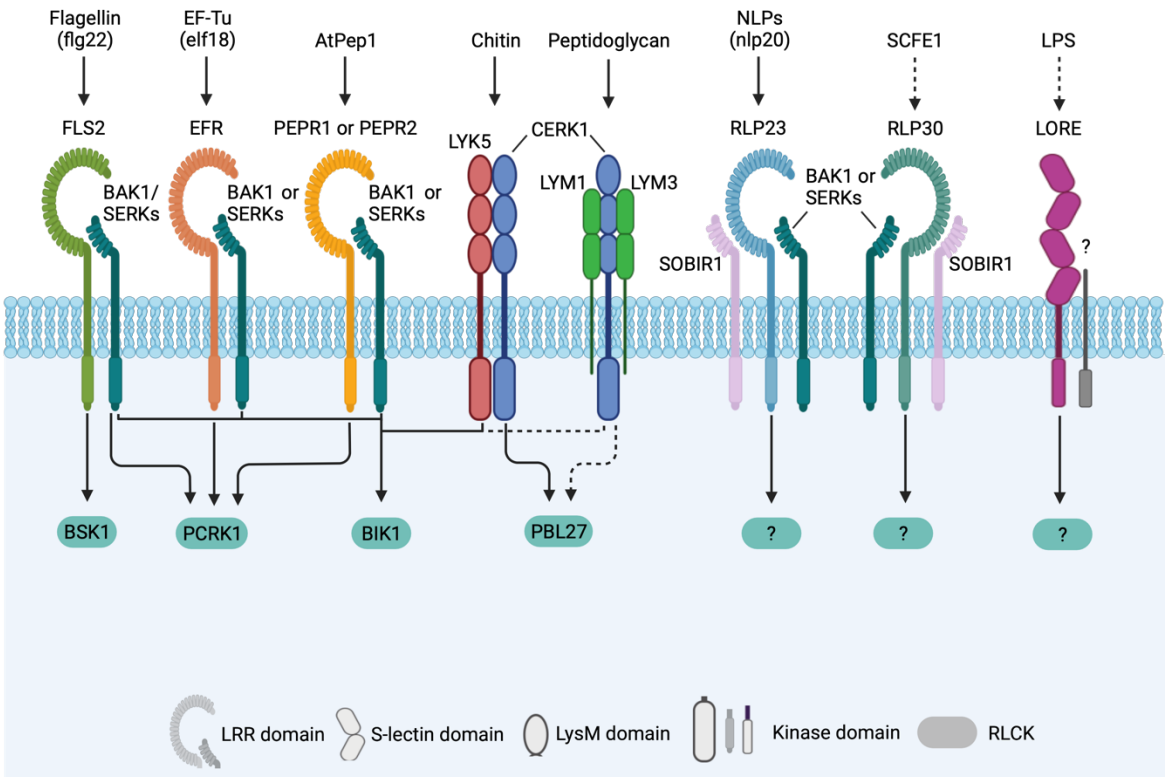



**Fig. 1.2 Arabidopsis receptor kinases and receptor-like cytoplasmic kinases (RLCKs).** Depending on the ectodomain, Pattern recognition receptors (PRRs) recruit distinct receptor kinases. RLCKs are also recruited to distinct PRR complexes. Figure adapted from Couto & Zipfel (2016), created with BioRender.com.

PAMPs perception by PRRs initiated on the cell surface through kinase complexes which often called as leucine-rich repeat receptor kinases (LRR-RKs) (Yeh et al. 2015). The examples of LRR-RKs with known functions are FLAGELLIN SENSING2 (FLS2) that involved in perception flagellin epitope, flg22 (Chinchilla et al. 2006) and EF-TU receptors (EFR) that recognize EF-Tu or the derived peptides, elf18 or elf26 (Yeh et al. 2015; Couto & Zipfel 2016). In addition to the receptors mentioned above, PRRs can also recognize damage-associated molecular patterns (DAMPs), the endogenous molecules that release upon abiotic stresses responses, i.e., wound (Saijo et al. 2018). The LRR-RKs PROPEP RECEPTOR1 (PEPR1) and PEPR2 are responsible for perceiving wound-induced endogenous peptide, PEP1 (Roux et al. 2011).

The perception of PAMPs by PRRs can trigger a chain of complex responses to arrest the pathogens invasion called PAMP-triggered immunity (PTI) (Bigeard et al. 2015; Qi et al. 2011; **Fig.**

**1.3**). PTI mostly symptomless, however in the molecular level extensive changes take place to prevent pathogens proliferation in the plant tissues (Szatmári et al. 2014). It is including activation of reactive oxygen species (ROS), callose depositions and activation of defense-related genes (Hatsugai et al. 2017; Ngou et al. 2021).

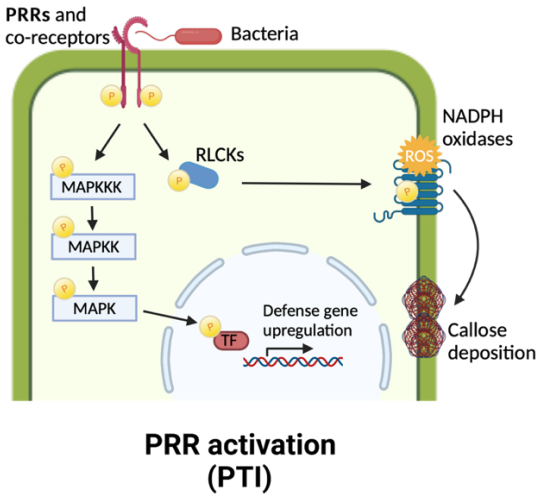



**Fig.1.3 PAMPs perception by PRRs activate PAMP-triggered immunity.** Upon PAMPs detection by PRRs, PTI leads to activation of co-receptors (e.g., BIK1), production of reactive oxygen species (ROS) through NADPH oxidases activation, activation of mitogen-activated protein kinases (MAPKs) and induction of defense-related genes. Figure adapted from Ngou et al. (2021), created with BioRender.com.

### 1.1.2 Effector-triggered immunity (ETI)

In response to plant PTI, adapted pathogens may release effectors, proteins which produced by plant pathogens to arrest plants defense and allow the pathogens proliferate in the plant tissues. Therefore, pathogens effector recognition by the plant is essential to trigger effector-triggered immunity (ETI). ETI is known as intracellular defense responses that activated after pathogen effectors perception by nucleotide binding-leucine rich repeat (NB- LRR) protein (Toruño et al. 2016). Furthermore, strong immune responses, including program cell death (PCD), can occur at the sites where the pathogens infected the plants which called as the hypersensitive response (HR) (Hatsugai et al. 2017). Popular illustration of the plant immune system is known as the “zig-zag” model (**Fig. 1.4**).

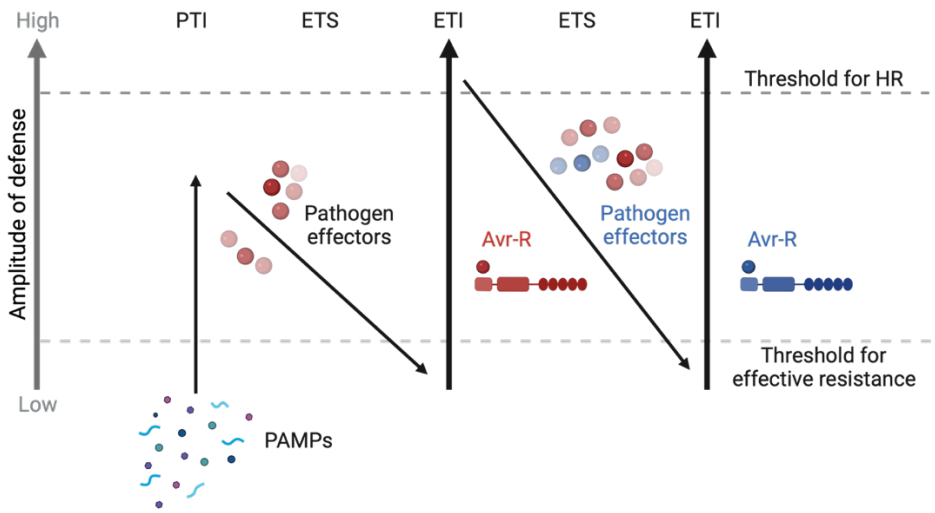



**Fig. 1.4 A zig-zag model illustrates the plant immune system.** In the first phase, detection of PAMPs by PRRs leads to activation of PTI. In the second phase, successful pathogens releases effectors to interfere plant PTI and allow the pathogens proliferate in the plant tissues, resulting in effector-triggered susceptibility (ETS). In the third phase, one pathogen effector (indicated in red) is recognized by NB-LRR protein, resulting in the activation effector-triggered immunity (ETI), a stronger version of PTI often followed by hypersensitive response (HR). In the fourth phase, pathogen isolates which have lost red effector, possibly gained new effector (indicated in blue) to help suppressing plant ETI. New plant NB-LRR alleles can recognize one of the newly acquired effectors, resulting in ETI once more. Figure adapted from Jones & Dangl (2006), created with BioRender.com.

Although ETI is an amplified version of PTI, ETI cannot work independently and requires PTI activation in order to provide effective pathogens resistance. Mutual potentiation of PTI and ETI has been reported enabling to a stronger immune response (Ngou et al. 2021). Thus, the mutual potentiation will further restrict pathogen proliferation and increase the plant resistance to disease.

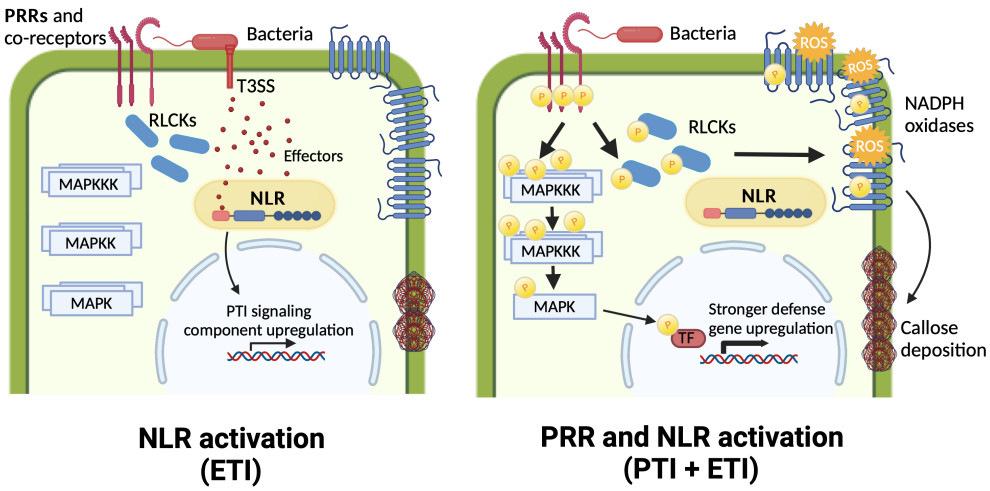



**Fig. 1.5 PTI and ETI co-activation.** Co-activation of PTI and ETI enhances the activation of PTI signal complexes, resulting in stronger defense response. Figure adapted from Ngou et al. (2021), created with BioRender.com.

### 1.1.3 Host and non-host resistance

Various abiotic and biotic stresses continually challenge plants. Biotic stresses caused by bacteria, fungi, oomycetes, and viruses are considered as the major threats (Gill et al. 2015). In nature, most of the plants are resistant to most of the microorganisms, and only a few of them can cause disease in plants (Stam et al. 2014).

There are two kinds of plant-disease resistance: host resistance and non-host resistance. Host and nonhost resistance are differentiated based on the pathogen capability to successfully infect certain species (host) or unsuccessfully infect other species (non-host) (Gill et al. 2015). Therefore, the pathogen cannot infect all plant species, and plants species are always vulnerable to specific pathogens (Gill et al. 2015; Shiraishi 2013).

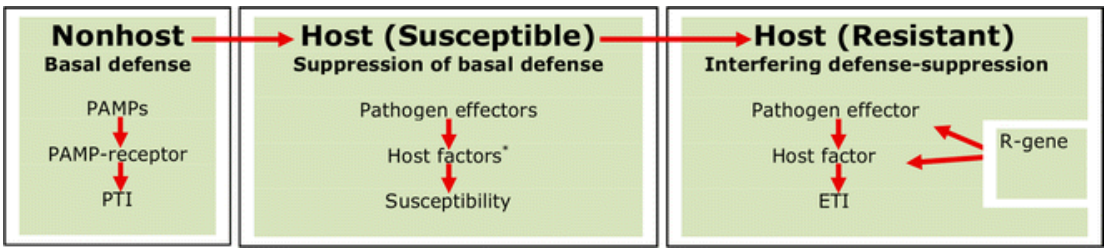



**Fig. 1.6 Illustration of plant host and non-host resistance.** PAMPs perception by PRRs leads to activation of PTI, contributing to nonhost resistance (basal defense). Pathogen effectors released by successful pathogens target host factors, resulting in host susceptibility. When pathogens effectors cannot exploit host factors, plant will continue to maintain the nonhost resistance. Detection of pathogens effectors by R-proteins resulting in plant ETI and when numerous R-genes for the same pathogen species are present in a plant, the combined ETI will also lead to nonhost resistance. Figure adapted from Seifi et al. (2014).

## 1.2 Endogenous suppressor (ES)

The plant-pathogen interaction is affected by pathogen-derived elicitors or suppressors (Shiraishi 2013). Elicitor is defined as a substance which elicits defense-responses, while suppressor is known to arrest elicitor activity (Keen 1975; Shiraishi 2013). The antagonistic activity between elicitor and suppressor may determine the plant resistance or susceptibility to various pathogen races (Keen 1975).

It has been previously reported that pea fungal pathogen, *Mycosphaerella pinodes* (*M. pinodes*) secreted suppressors from spore germination fluid (Shiraishi 2013). The suppressors, called as supprecins A and B, can block elicitor-induced defense responses such as the production of

phytoalexin, activation of ROS, and activation of pathogenesis-related (PR) proteins (Shiraishi 2013). Furthermore, exogenous application of *M. pinodes* suppressors allowed non-adapted fungal pathogens successfully infect pea plant. Also, application of *M. pinodes* suppressors along with *Altenaria alternata* (Japanese pear pathogen) allow the non-adapted fungal pathogen to infect several plant-species. Accordingly, suppressor-induced susceptibility activity has been reported strictly species-specific (Shiraishi 2013).

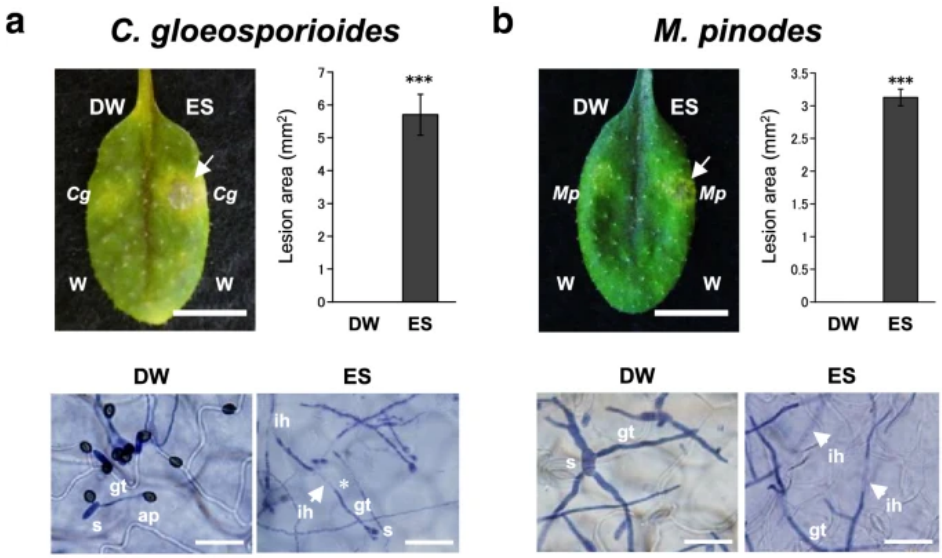



**Fig. 1.7 Induction of susceptibility on Arabidopsis Col-0 leaves in the presence of ES.** Partially purified ES induces susceptibility in Arabidopsis Col-0 challenged with *Colletotrichum tropicale* (previously reported as *Colletotrichum gloeosporioides* S9275) and *M. pinodes*. Figure adapted from Mai et al. (2020).

Infection enhancing factor (IEF) has been previously reported present in healthy barley leaves (Shiraishi et al. 1989). Interestingly, IEF from healthy barley leaves can enhance the infection of the incompatible race of powdery mildew fungus, *Erysiphe graminis* H1 (Shiraishi et al. 1989). The similar IEF also found in healthy wheat (Shiraishi et al. 1989), pea seedlings (Nasu et al. 1992), and Komatsuna (*unpublished data*). These findings suggest that plants may commonly produce IEF to determine the plant resistance or susceptibility towards pathogen (Shiraishi et al. 1989). The IEF tentatively called as an endogenous suppressor (ES), and ES from healthy pea seedlings has been reported enhance susceptibility in pea plant towards non- adapted pathogens (Nasu et al. 1992).

From our previous study, we confirmed the presence of ES in the model plant *Arabidopsis thaliana* ecotype Col-0 and the partially purified ES preparation induces susceptibility in Arabidopsis against non-adapted *M. pinodes* (*Mp*) and *Colletotrichum tropicale* (*Ctro*; previously reported as

*Colletotrichum gloeosporioides* S9275) (Mai et al. 2020; **Fig. 1.7**). Similar to the pea ES, the Arabidopsis preparation severely inhibited PAMP-induced defense responses, i.e., ROS production and expression of defense-related genes (Mai et al. 2020). In the course of characterizing the Arabidopsis ES, we found that the Arabidopsis c-terminally encoded peptide (CEP) is one of the possible candidates of ES.

## 1.3 C-terminally encoded peptide (CEP) family in Arabidopsis

CEP peptide was discovered relatively recently through an *in-silico* approach (Ohyama et al., 2008). Through a bioinformatics approach, a total of 15 members of CEP family (CEP1 to CEP15) were identified in *Arabidopsis thaliana* (*A. thaliana*) and the genes encoding CEP peptides are spread over different chromosome, except chromosome 4 (Roberts et al. 2013). CEP genes distribution is conserved among seed plants but not in lower land plants and green algae (Roberts et al. 2013; Delay et al. 2013). Also, CEP genes expression can be found throughout Arabidopsis plant (**Fig. 1.8**) and affected by environmental cues, i.e., nitrate limitation (Roberts et al. 2013).

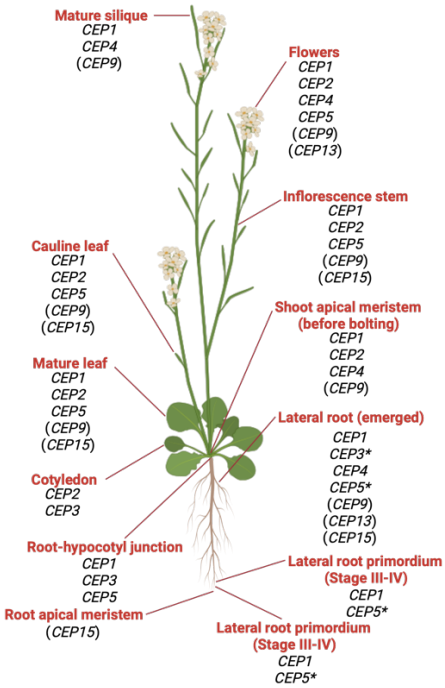



**Fig. 1.8 Expression of *CEP* genes in *A. thaliana*.** CEP genes are expressed throughout Arabidopsis plant. Figure adapted from Roberts et al. (2013), created with BioRender.com.

Individual members of CEP peptide-encoding genes, encoding proproteins containing one to five CEP domains at or near the C-terminus, yielding small peptides consisting of 15 amino acids through post-translational modifications (Ohyama et al. 2008; Delay et al. 2013; Taleski et al. 2018;

**Fig. 1.9**). The resulting CEP peptides are usually hydroxylated on the proline residues through a putative hydroxylase (Ohyama et al. 2008; Tabata et al. 2014; Mohd-Radzman et al. 2015, Eves-Van Den Akker et al. 2016). The hydroxyprolination is expected affect CEP peptide bioactivity and hydrophilic nature (Roberts et al. 2013).

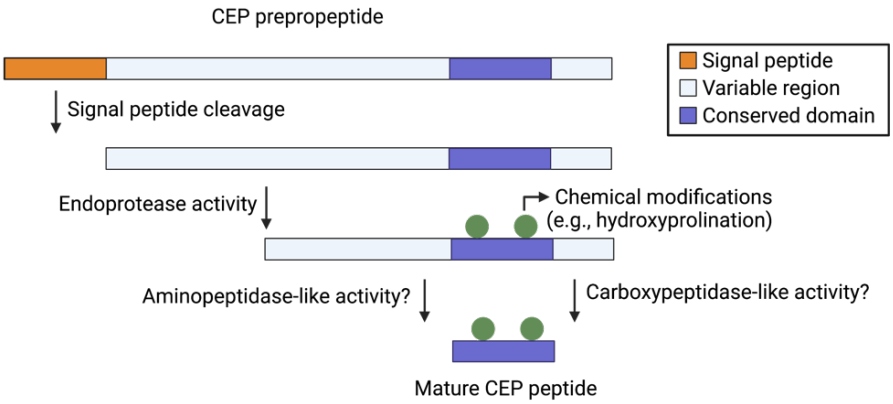



**Fig. 1.9 CEP peptide post-translational modifications.** CEP precursor protein (CEP prepropeptide) comprises of a signal peptide and at least one conserved CEP domain. After signal peptide cleavage, CEP prepropeptide undergoes few modifications in the apoplast, including peptide length, sequence, and chemical modifications, resulting in biologically active 15-amino-acid peptides. Figure adapted from Taleski et al. (2018), created with BioRender.com.

Although CEP peptides belong to one peptide family, within the family, CEP peptides are divided into two groups based on CEP domain sequence similarity (**Fig. 1.10**). Group I consists of CEP1-12 whilst Group II consists of CEP13-15 (Roberts et al. 2013). As some amino acids in the C-terminus part of the CEP domain showed high similarity in both groups (SPGV/IGH) and the amino acid sequence within the group is well conserved, there is a possible redundant function as well within each group (Roberts et al. 2013).

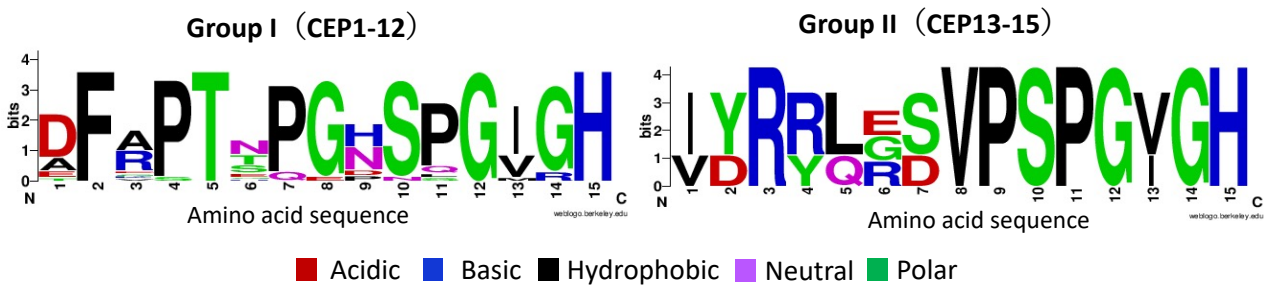



**Fig. 1.10 The two groups of CEP family.** CEP peptides are divided into two groups based on CEP domain sequence similarity. Figure adapted from Roberts et al. (2013), created with BioRender.com.

CEP peptide(s) is well characterized as a root-to-shoot signal molecule that is generated in roots that sense nitrogen (N) deficiency and subsequently compensates for N acquisition through interaction with typical LRR-type receptor kinases CEPR1/XIP1 and CEPR2 in the shoot vascular tissues (Tabata et al. 2014; Ohkubo et al. 2017). CEP peptide(s) interaction with CEPR1/XIP1 enabling upregulation of two phloem-mobile polypeptides, CEP downstream1 (CEPD1) and CEPD2, which relay the signal in a root-ward direction to upregulate *NRT2.1* expression in high-N-exposed roots (Tabata et al. 2014; Ohkubo et al. 2017; Taleski et al. 2018). In addition, Roberts et al. (2016) showed that besides CEPR1/XIP1 known role in N acquisition, CEPR1/XIP1 also known to interact with CEP5 to control lateral root initiation in Arabidopsis (**Fig 1.11**).

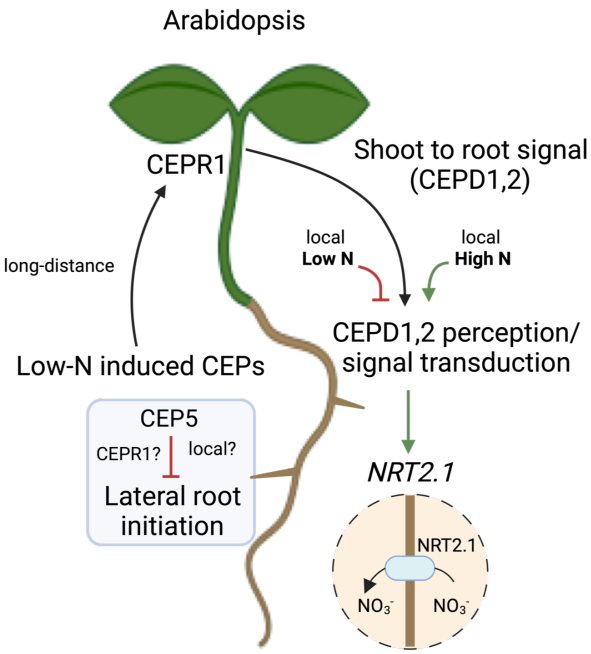



**Fig. 1.11 CEP peptide interaction with CEP receptor(s) (CEPR) in *A. thaliana*.** CEP-CEPR1/XIP1 interaction in the shoot leads to upregulation of CEPD polypeptide. These polypeptides move in a root-ward direction then upregulate NRT2.1 in high-N-roots to compensate N-acquisition in low-N-roots. Through CEPR1/XIP1, CEP5 inhibits lateral root initiation. Figure adapted from Taleski et al. (2018), created with BioRender.com.

## 1.4 Objectives

CEP peptide(s) is well characterized as a root-to-shoot signal peptide that is generated in roots that sense nitrogen (N) deficiency and subsequently compensates for N acquisition from nutrient-rich roots through systemic signaling (Delay at al. 2013; Ohkubo et al. 2017; Ohyama et al. 2008; Roberts et al. 2013). However, hardly anything is known about its activity for defense suppression. Therefore, the general objective of this study is to elucidate CEP peptide role in plant immunity. First, we aimed to investigate the effect of CEP5p$^{hyp}$ on Arabidopsis immunity against fungal and bacterial pathogens. Second, we aimed to investigate whether the CEP5p$^{hyp}$ suppresses or delays the Arabidopsis immune responses. Finally, to understand the role of CEP peptides in modulating the Arabidopsis immunity, expression for *CEP* genes (*CEP1* to *CEP15*) were analyzed with Col-0 leaves challenged with bacterial pathogens. It has been previously reported that exogenous application of synthetic CEP5p$^{hyp}$ can arrest primary and lateral root growth in Arabidopsis. Because of this, we hypothesized that CEP peptides would prevent excessive defenses by fine-tuning the trade-off between the growth and the defense.

# Chapter 2: CEP peptide induces susceptibility of *Arabidopsis thaliana* to non-adapted pathogens


## Abstract

CEP peptide allowed non-adapted pathogens to cause symptoms, when applied exogenously to Arabidopsis Col-0. *Colletotrichum tropicale* (*Ctro*) could not infect the Col-0, but in the presence of CEP peptide, the conidia germinated to form hyphal-like structures, which directly penetrate epidermis without forming melanized appressoria. This is reminiscent of the hyphal tip-based entry (HTE) reported by Hiruma et al. (2010) showing that *Ctro* employs the HTE on Arabidopsis *pen2* mutant. Intriguingly, the CEP peptide-mediated susceptibility was observed on Arabidopsis mutants lacking the CEP peptide receptors, CEPR1/2. Our observations indicate that CEP peptide likely modulate Arabidopsis immune response through an unidentified receptor(s).



**Keywords:** Arabidopsis immunity, c-terminally encoded peptide (CEP), Endogenous suppressor (ES), defense suppression, induced susceptibility.

## 2.1 Introduction

An endogenous suppressor (ES) is defined as constitute(s) synthesized by plant, which suppress(es) elicitor-induced defense responses when applied to the plant (Nasu et al. 1993; 1995). The role of ES is considered to prevent excessive defense responses and fine-tune the trade-off between the growth and the defense (Nasu et al. 1993). Recently, Arabidopsis ES was shown not only to suppress PAMP-induced defense responses, but also to induce susceptibility to non-adapted pathogens (Mai et al. 2020). Further LC-MS/MS analysis of purified Arabidopsis ES identified a peptidic fragment corresponding to a c-terminally encoded peptide 5 (CEP5).

CEP peptide(s) is well characterized as a root-to-shoot signal peptide that is generated in roots that sense nitrogen (N) deficiency and subsequently compensates for N acquisition from nutrient-rich roots through systemic signaling (Delay at al. 2013; Ohkubo et al. 2017; Ohyama et al. 2008; Roberts et al. 2013). In Arabidopsis, the CEP peptide-encoding genes comprise a large family (*CEP1* to *CEP15*), with individual members encoding proproteins containing one to five CEP domains at or near the C-terminus, yielding small peptides consisting of 15 amino acids through post-translational modifications (Ogilvie et al. 2014; Roberts et al. 2013). The resulting CEPs are usually hydroxylated on the proline residues through a putative hydroxylase (Imin et al. 2013; Mohd-Radzman et al. 2015; Roberts et al. 2013; Taleski et al. 2018).

Under low N condition, root-derived CEP peptides migrate to the vascular tissue of the shoot and interact with typical LRR-type receptor kinases CEPR1/XIP1 and CEPR2, which then transduce N deficiency systemically into roots favorable for N acquisition via a newly synthesized shoot-root signal (Ohkubo et al. 2017; Tabata et al. 2014). Additionally, the CEP peptides locally restrict the growth of primary roots and promotes the lateral root formation (Delay et al. 2013; Roberts et al. 2013; Smith et al. 2020). In *Medicago truncatula* under high nitrogen conditions, a certain CEP peptide, whose is induced in response to symbiotic association or Nod factor-treatment, regulate locally or systemically secondary infection of rhizobia via affecting ethylene-mediated signaling pathway (Zhu et al. 2020). Therefore, it is becoming clear that CEP peptides regulate not only plant growth, but also their association with beneficial microorganisms as symbiotic interaction can be costly for plants. In this communication, we investigate the effect of synthesized CEP peptide on plant immunity, especially on its induction of disease susceptibility to non-adaptive pathogens, using Arabidopsis plants.

## 2.2 Materials and methods

Seeds of *Arabidopsis thaliana* Col-0 were sown on wetted Jiffy-7 peat pellets (AS Jiffy Products, Oslo, Norway), grown at 22°C in a growth chamber with a 10 h light/14 h dark cycle at 11.8 $W \cdot m^{-2}$ for two weeks. Germinated seeds were transplanted into 1:1 mixture of soil (Sakata Seed Co., Ltd., Yokohama, Japan) and vermiculite and grown under the same condition as mentioned above for additional 2-3 weeks before use. The Arabidopsis lacking CEPR1 and CEPR2; cepr*1-1*, *cepr2-1* and *cepr1-1 cepr2-1* from the Nossen (No-0) (Ohkubo et al. 2017; Tabata et al. 2014) and an additional *CEPR1* knock-out allele, *cepr1-3* from the Col-0 (Chapman et al. 2020) were obtained from the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan and from the Nottingham Arabidopsis Stock Centre (NASC), respectively, and were used to evaluate the role of CEPR1 and CEPR2 in the CEP5p$^{hyp}$-mediated disease susceptibility.

Synthetic CEP5p$^{hyp}$ (DFR{HYP}TT{HYP}GHS{HYP}GIGH) (>98% purity) was synthesized by GL Biochem Pty Ltd. (Shanghai, China). For CEP5p$^{hyp}$-mediated disease susceptibility in Arabidopsis, a conidial suspension of non-adapted *Colletotrichum tropicale* (*Ctro*) strain S9275 was drop-inoculated together with CEP5p$^{hyp}$ solution onto the adaxial surface of detached leaves from 5-week-old Arabidopsis Col-0. The inoculated leaves were kept in a plastic tray filled with wetted tissue paper and incubated at 22°C in a dew chamber with a 10 h light/14 h dark cycle at 11.8 $W \cdot m^{-2}$. Disease symptoms were observed 3 days post inoculation (dpi). For the short-term treatment of CEP5p$^{hyp}$, the adaxial side of detached leaves was pretreated for 3 h, and the conidial suspension was then inoculated on the same place after removing the peptide solution. For inoculation with *Pseudomonas syringae* pv. *tabaci* strain 6605 (*Pta* 6605) (Taguchi et al. 2006), four fully expanded leaves of 5-week-old Arabidopsis Col-0 were syringe-infiltrated with CEP5p$^{hyp}$ 3 h before inoculation with the bacterial suspension. The inoculum density was adjusted to 4 x $10^6$ cfu/ml (equiv. $OD_{600}$ = 0.002). The inoculated plants were kept under high humidity in a plastic container with a clear lid. Disease symptoms were observed at 3 and 5 dpi. For measuring bacterial population, the inoculated leaves were collected at 0, 3 and 5 dpi. Serially diluted samples were placed on King's B (KB) agar plates containing nalidixic acid antibiotic (50 µg/ml). After incubation at 27°C for 2 days, the number of bacterial colonies was counted using the selected dilution series that could be measurable accurately and evaluated as colony forming units (cfu) per $cm^2$ of leaves.

For quantitative RT-PCR (qRT-PCR) analysis, a conidial suspension of *Ctro* was inoculated on three fully expanded leaves in the absence or presence of CEP5p$^{hyp}$. Inoculated leaves were collected at 9 h post-inoculation (hpi) from three different plants. Total RNA was reverse transcribed as

previously described (Mai et al. 2020; Phuong et al. 2020). The qRT-PCR was performed with a Shimadzu GVP-9600 Gene Detection System (Shimadzu, Kyoto, Japan), using a Toyobo Thunderbird Next SYBR qPCR mix (Toyobo, Tokyo, Japan). Primers were listed in supplementary Table S2.1 and *EF1-α* (At5g60390) was used as a reference gene.

## 2.3 Results and discussions

First, to investigate whether CEP5p$^{hyp}$ exerts an inhibitory effect on morphogenesis during the invasive stage of *Ctro*, we observed its infection-related behaviors such as appressorial differentiation and subsequent formation of infection hyphae from the appressoria by contacting the conidia with the CEP5p$^{hyp}$ on ethanol-killed onion epidermis. As shown in **Fig. S2.1a**, *Ctro* germinated to differentiate melanized appressoria by 8 hpi, which then extended infection hyphae by 12 hpi even in the presence of CEP5p$^{hyp}$ (**Fig. S2.1a**). Thus, no detrimental effect on *Ctro* caused by CEP5p$^{hyp}$ was observed (**Fig. S2.1a**).

We next investigated whether CEP5p$^{hyp}$ can induce susceptibility in Arabidopsis against non-adapted *Ctro*, similar to Arabidopsis ES. While inoculation of the non-adapted *Ctro* conidia did not induce disease symptom on Col-0 leaves, symptoms were developed when the conidia were inoculated simultaneously with the CEP5p$^{hyp}$ (**Fig. 2.1a**). Furthermore, although the size of the lesions formed was small, induction of susceptibility to *Ctro* was shown to be effective even with a short-term treatment of 3 h before inoculation (**Fig. 2.1b**).

We then observed the lesions induced by *Ctro* under a light microscope. Interestingly, our observation revealed that the conidia germinated to form appressoria, which attempted to penetrate on the control leaves, but failed to extend infection hyphae in the absence of CEP5p$^{hyp}$ (**Fig. 2.1c**). In contrast, in the presence of CEP5p$^{hyp}$, the conidia extended hyphal-like structures, then directly penetrated into epidermal cells without forming melanized appressoria (**Fig. 2.1c**). This observation is reminiscent of the hyphal tip-based entry (HTE) reported by Hiruma et al. (2010), who showed that *Ctro* employs the HTE on Arabidopsis mutant lacking *PEN2* or on the wild-type Col-0 exposed to heat treatment. Thus, exogenous CEP5p$^{hyp}$ may alter the physiology of Arabidopsis cell to a susceptible state against the non-adapted pathogens, like in the *pen2* mutant and heat-treated Col-0. Indeed, CEP5p$^{hyp}$ also induced susceptibility to non-related *Mycosphaerella pinodes* (*Mp*), a pea pathogen, causing disease symptoms on Col-0 leaves (**Fig. S2.2a, b**).

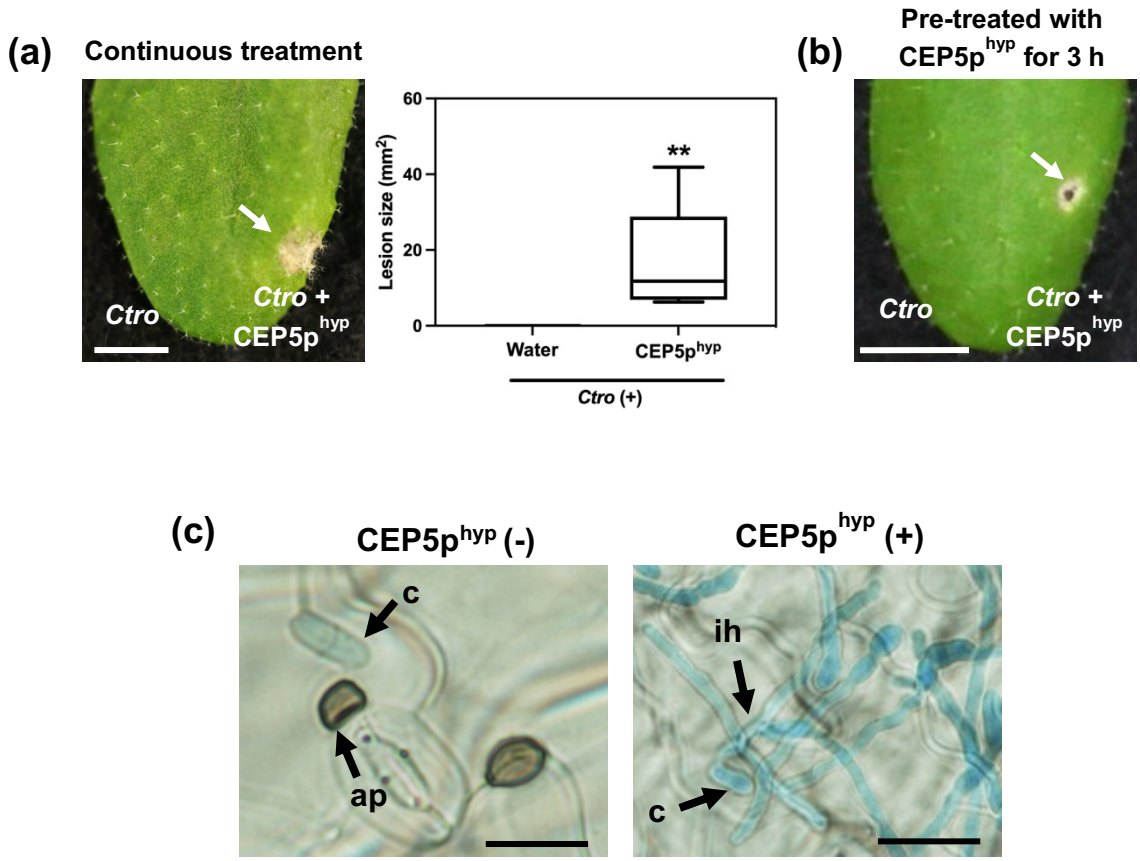



**Fig. 2.1 CEP5p$^{hyp}$ induces susceptibility in Arabidopsis Col-0 when inoculated with non-adapted *Colletotrichum tropicale* (*Ctro*) (a)** Mature leaves of 5-week-old Arabidopsis Col-0 were drop-inoculated with conidial suspension (5 x $10^5$ conidia/ml) of *Ctro* in the presence of distilled water as a control or synthetic CEP5p$^{hyp}$ (1 mM). Arrows indicate lesions developed on the Arabidopsis leaves. Photos were taken at 3 dpi. Scale bar = 1 cm. Lesion size was measured using Fiji/ImageJ (Wayne Rasband, NIH). The means and standard deviations (SDs) were calculated from three independent experiments (n=3). Asterisks indicate significant difference from the control using two-tailed Student's *t* test (** $p < 0.002$). Statistical analyses were performed with GraphPad Prism 9.1.2 (GraphPad Software, Inc., San Diego, CA). **(b)** Arabidopsis Col-0 leaves pre-treated with CEP5p$^{hyp}$ for 3 h developed disease symptoms after inoculated with *Ctro*. **(c)** Microscopic observation revealed that in the presence of CEP5p$^{hyp}$, *Ctro* developed intracellular hyphae (ih) from the tip of the hyphae without forming melanized appressoria, which had been described as the hyphal tip-based entry (**HTE**; Hiruma et al. 2010). Scale bar = 50 µm. **c**, conidiospore. **ap**, appressoria. **ih**, infection hyphae.

Previously, Mai et al. (2020) reported using *M. pinodes* as a non-adapted fungal pathogen, that adding the Arabidopsis ES caused more severe symptoms on *jar1* plant, compared to the wild-type Col-0. To gain insight in the CEP5p$^{hyp}$-mediated disease susceptibility, we performed a similar assay using the *jar1-1* (Staswick and Tiryaki 2004). In line with our previous result (Mai et al. 2020), *M.*

*pinodes* could not infect *jar1-1* plant, while in the presence of CEP5p$^{hyp}$, *jar1-1* was more susceptible than Col-0 (**Fig. S2.2a, b**). This result is consistent with that exogenous CEP peptide likely attenuates the first layer of Arabidopsis resistance to *M. pinodes*, enabling to grow more vigorously in *jar1* plants, as it is a hemi-biotrophic pathogen.

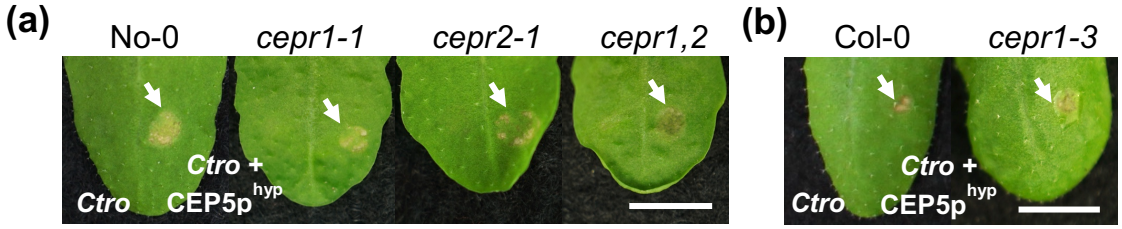



**Fig. 2.2 CEP5p$^{hyp}$ induces susceptibility to non-adapted *Ctro* in Arabidopsis *cepr* mutant plants lacking CEP peptide receptors, CEPR1 and CEPR2 (No-0 or Col-0 background). (a)** Detached leaves of 5 weeks old Arabidopsis No-0, *cepr1-1*, *cepr1-2* and *cepr1,2* were inoculated with conidial suspension of *Ctro* (5 x $10^5$ conidia/ml) in the presence of CEP5p$^{hyp}$ or water as control treatment. Arrows indicate lesions developed on the Arabidosis leaves. Photos were taken at 3 dpi. Scale bar = 1 cm. **(b)** Detached mature leaves of Col-0 and *cepr1-3* were inoculated with *Ctro* conidial suspension in the presence of CEP5p$^{hyp}$ or distilled water.

To investigate whether susceptibility induction by CEP5p$^{hyp}$ is mediated by CEPR1 and CEPR2, the *cepr1-1*, *cepr2-1* and *cepr1-1 cepr2-1* mutants (No-1 background) were tested for induction of susceptibility using *Ctro* as a test fungal pathogen. The wild-type No-0 was used as the control. As shown in **Fig. 2.2a**, the CEP5p$^{hyp}$-mediated susceptibility was visible on the No-0 and *cepr* mutant plants when inoculated with *Ctro*. An additional *cepr1-3* with a different mutation allele in the Col-0 background also showed the same phenotype (**Fig. 2.2b**). A similar result was also observed on No-0 and *cepr* mutant plants when inoculated with *Mp* (**Fig. S2.3a-b**). Taken together, these results suggest that signaling pathway underlying the CEP5p$^{hyp}$-induced susceptibility is mediated independently of CEPR1 and CEPR2, which are known to act on systemic signaling during N-deficiency. Rather, an unidentified receptor(s) other than CEPR1 and CEPR2 is likely involved in the CEP5p$^{hyp}$-mediated disease susceptibility.

Hiruma et al. (2010) previously reported that non-adapted *Ctro* switches to the HTE mode and the resulting hyphae directly extended in Arabidopsis leaves lacking *PEN2* and *PEN3*, eventually causing disease symptoms. Those two genes are known to be involved in preinvasive resistance to restrict the *Ctro* entry into Arabidopsis epidermal cells (Kosaka et al. 2020; Hiruma et al. 2010). Furthermore, even *Ctro* successfully penetrates into epidermal cells of *pen2* and *pen3* mutants,

postinvasive resistance would be activated to restrict further *Ctro* invasion, which involves antimicrobial camalexin (Kosaka et al. 2020; Pastorczyk et al. 2019).

To investigate whether CEP5p$^{hyp}$ suppresses preinvasive resistance, which is associated with *PEN2* and *PEN3*, to allow *Ctro* successful infection in the Col-0, we conducted qRT-PCR analysis. In line with previous report (Hiruma et al. 2010), our qRT-PCR analysis revealed that *PEN2* was induced upon *Ctro* inoculation (**Fig. 2.3a**). However, *PEN2* expression level induced by *Ctro* inoculation was significantly suppressed in the presence of CEP5p$^{hyp}$ (**Fig. 2.3a**). Unexpectedly, opposite to the *PEN2* expression result, *PEN3* expression was induced in the presence of CEP5p$^{hyp}$ when inoculated together with *Ctro* although the expression level almost the same with mock-inoculated plants (**Fig. 2.3b**). These findings suggest that exogenous CEP5p$^{hyp}$ possibly targets at least *PEN2*-, but not *PEN3*-dependent preinvasive resistance in Arabidopsis.

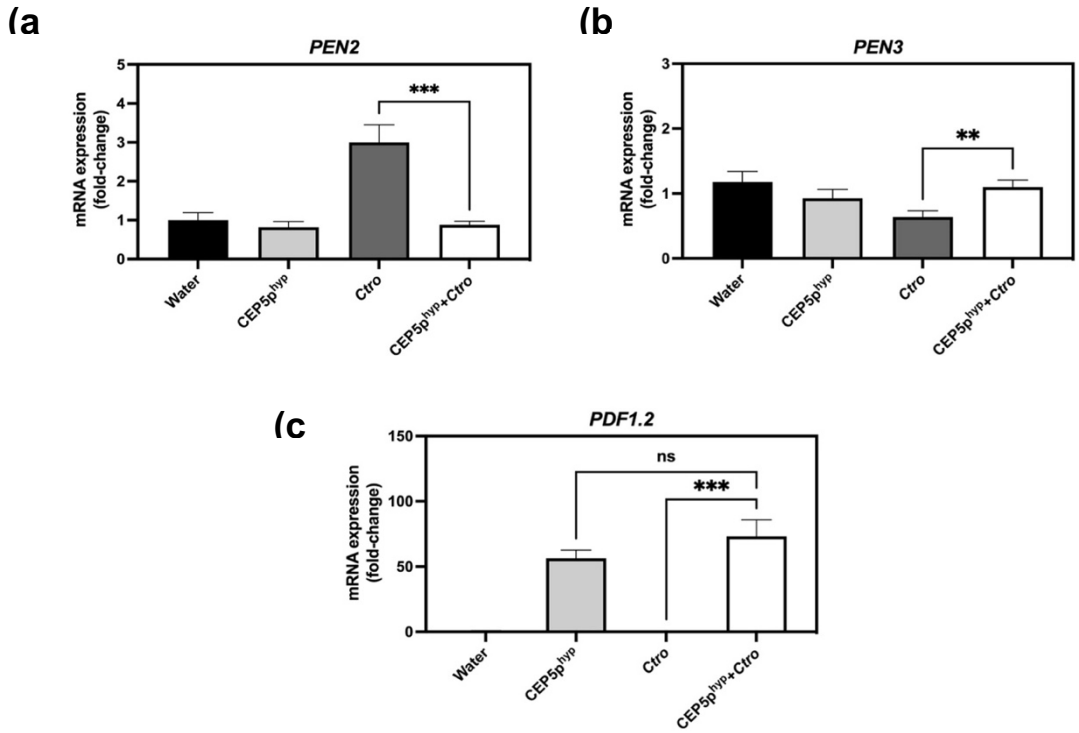



**Fig. 2.3 Expression of *PEN2*, *PEN3* and *PDF1.2* upon *Ctro* inoculation in the presence of CEP5p$^{hyp}$.** Five-week-old Arabidopsis Col-0 plants were drop-inoculated with conidial suspension of *Ctro* (5 x $10^5$ conidia/ml) in the presence or absence of CEP5p$^{hyp}$ (1 mM). Distilled water was used as the control. Relative expression of *PEN2*, *PEN3* and *PDF1.2* was quantified at 9 hpi by qRT-PCR. Expression level was normalized to the level of *EF1-α*. Values are means of three biological replicates and three technical replicates repeated twice. Error bars represent standard errors (SEs). Asterisks indicate significant differences among treatments using two-way ANOVA followed by Tukey's multiple comparison test (ns $p > 0.12$; ** $p < 0.002$; *** $p < 0.001$).

In contrast to those two genes, *PDF1.2* was drastically induced in Col-0 plants upon inoculation with the mixture of *Ctro* and CEP5p$^{hyp}$ (Fig 2.3c), whose expression level is quite similar to that of the *PDF1.2* in *pen2* mutants when invaded by *Ctro* at 17 hpi (Kosaka et al. 2020). Compared with water treated plants, *PDF1.2* expression was also induced by CEP5p$^{hyp}$ even without *Ctro* inoculation (Fig 2.3c). Indeed, the expression level was the same as that in Col-0 leaves challenged with *Ctro* with CEP5p$^{hyp}$, compared to that in Col-0 leaves without CEP5p$^{hyp}$. These results indicate that when PEN2-dependent preinvasive resistance, which limits *Ctro* entry, is suppressed by CEP5p$^{hyp}$ treatment, postinvasive resistance accompanied by expression of *PDF.1.2* is induced immediately upon its HTE-mediated invasion (Kosaka et al. 2020; Hiruma et al. 2010). Overall, our results suggest that the suppression of *PEN2* by CEP5p$^{hyp}$ during the *Ctro* infection in turn triggers the expression of *PDF1.2* gene inside the invaded cells as an additional layer of postinvasive resistance.

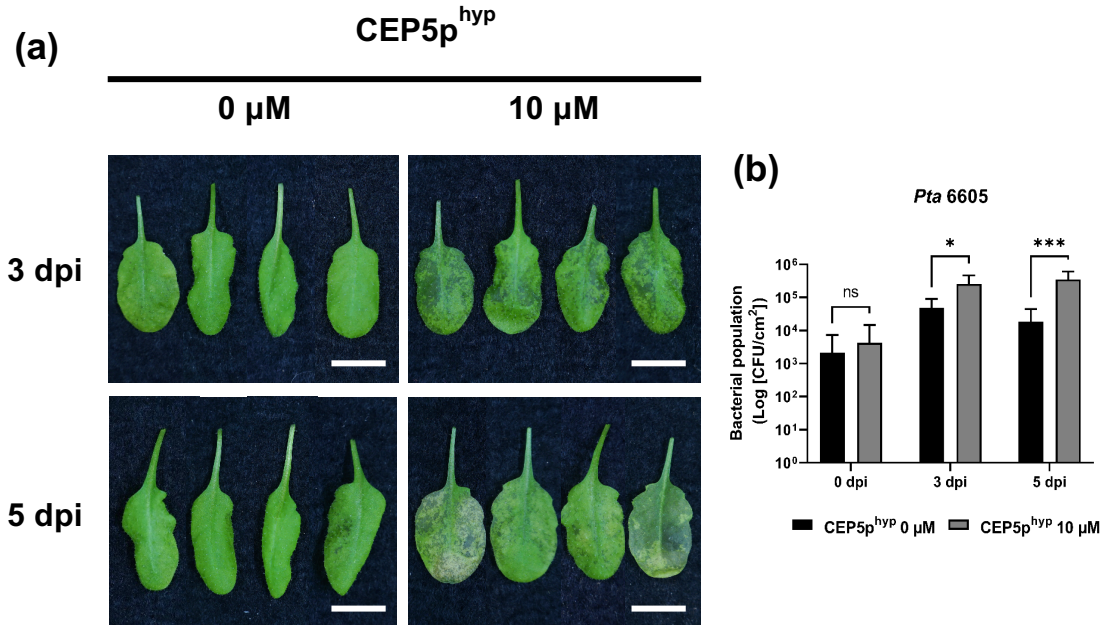



**Fig. 2.4 CEP5p$^{hyp}$ induces susceptibility in Col-0 against *Pseudomonas syringae* pv. *tabaci* 6605 (*Pta* 6605). (a)** Disease phenotype of syringe-infiltrated Arabidopsis Col-0 at 7 dpi. Arabidopsis Col-0 leaves (5 weeks-old) were syringe-infiltrated with a bacterial suspension *Pta* 6605 ($OD_{600}$: 0.002) after pre-treated with CEP5p$^{hyp}$ at 10 μM for 3 hours. (Scale bar = 1 cm). **(b)** Bacterial population of *Pta* 6605 in Arabidopsis Col-0. Bacterial populations were quantified at 0, 3 and 5 dpi. The means and SDs were calculated from three independent samples. The experiment was repeated four times, with similar results. Representative results are shown. Asterisks indicate significant differences from water treatment using two-way ANOVA followed by Sidak's multiple comparison test (***ns*** $p > 0.12$; * $p < 0.033$; ** $p < 0.002$).

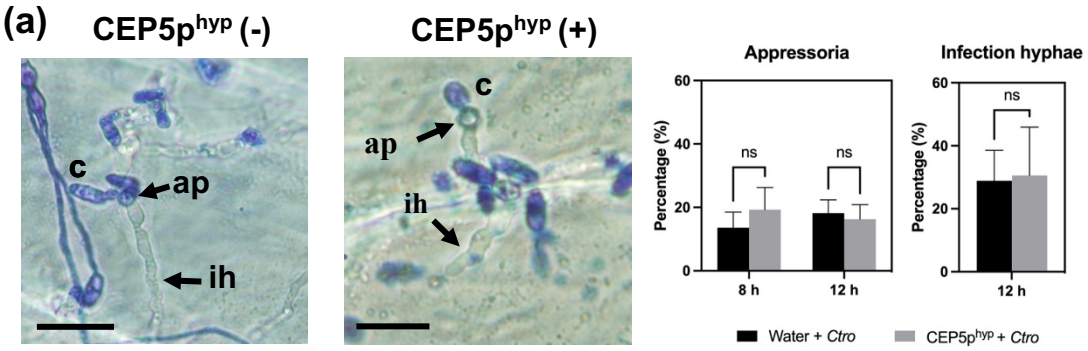
Finally, to find out whether the CEP5p$^{hyp}$-mediated susceptibility is effective against other non-adapted bacterial pathogen, we inspected CEP5p$^{hyp}$-mediated susceptibility on Col-0 challenged by *Pta* 6605. As shown in **Fig. 2.4a**, the Col-0 leaves pre-treated with CEP5p$^{hyp}$ showed disease symptoms by 3 days post syringe-infiltration with *Pta* 6605. The growth of *Pta* 6605 at 3 and 5 dpi significantly higher in Arabidopsis leaves with 10 μM CEP5p$^{hyp}$ compared with the mock-treated leaves (**Fig. 2.4b**). Since the CEP5p$^{hyp}$ did not affect the bacterial growth in *in vitro* (Fig. 1Sb), our observation suggests that exogenous CEP5p$^{hyp}$ can suppress the Arabidopsis immunity via probably affecting the common pathway(s) or process(es) leading to establishment of defense responses.

In this study, we demonstrated that similar to Arabidopsis ES, synthesized CEP peptide induced disease susceptibility to non-adapted pathogens including fungal and bacterial pathogens, when applied exogenously to the wild-type Col-0 (**Fig. 2.1**; **Fig. 2.4**). In addition, we showed that exogenous CEP5p$^{hyp}$ could prevent the expression of defense-related gene(s) associated with the preinvasive resistance such as *PEN2* gene (**Fig. 2.3a**). Notably, induced susceptibility to non-adapted pathogens were observed in the CEPR-deficient mutants essential for compensatory response during the N deficiency (**Fig. 2.3**). It is thus conceivable that the signaling underling the CEP-mediated susceptibility is independently from that initiates a compensatory response during N deficiency. Our present data thus enabled us to speculate that a yet-identified receptor(s) that induce susceptibility is different from the CEPRs that transduce(s) the N deficiency. In this scenario, individual CEP peptides from a huge CEP gene family (encoding at least 24 peptides), likely play diverse role(s) in various cellular processes, i.e., growth, symbiotic association, and immunity. Further studies on the signaling are expected to reveal more about CEP5p$^{hyp}$-mediated susceptibility.

## 2.4 Supplementary information

**Table S2.1** Primer sequences used for qRT-PCR

| Gene | Accession | Sequence (5' > 3') | Ref. |
|---|---|---|---|
| ***EF1-α*** | *At5g60390* | F: CATCATTTGGCACCCTTCTT<br>R: TGGTGACGCTGGTATGGTTA | Phuong et al., 2020 |
| ***PEN2*** | *At2g44490* | F: TAACATGCTTCTAGCGCACGCAG<br>R: CATCTGGATCACTCGGATCATATG | Hiruma et al., 2010 |
| ***PEN3*** | *At1g59870* | F: GGTGTTAAGAACAGTCTCGTCAC<br>R: TCTTCTGACCTCCAGATATACC | Hiruma et al., 2010 |
| ***PDF1.2*** | *At5g44420* | F: TCACCCTTATCTTCGCTGCTC<br>R: ATGTCCCACTTGGCTTCTCG | Jia et al., 2016 |



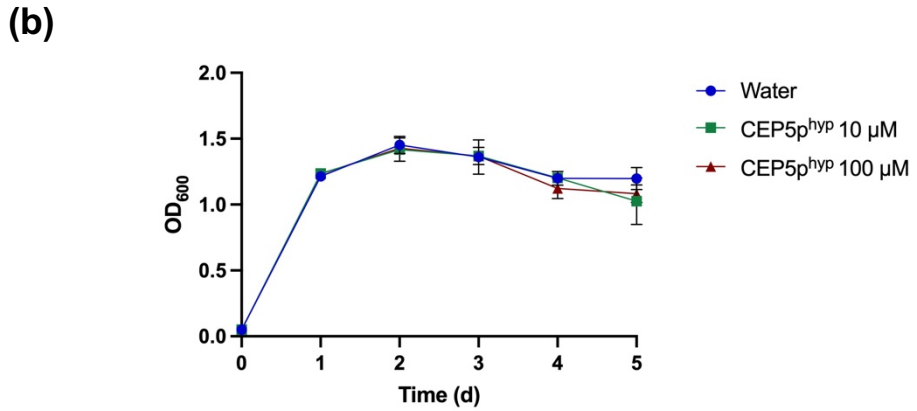




**Fig. S2.1 CEP5p$^{hyp}$ does not change *Ctro* infection behavior when inoculated on ethanol-killed onion epidermal strips.** Inner part of onion epidermis was cut into 1 cm$^2$ strips, immersed overnight in absolute ethanol, then washed extensively with distilled water before use. Conidial suspension of *Ctro* (5 x 10$^5$ conidia/ml) were spotted on the hydrophobic side of epidermal strips along with CEP5p$^{hyp}$ at 1 mM (final conc.) or with distilled water as a control (10 µl in total per strips). Onion epidermal strips were observed using an Olympus BX60 light microscope at 8 and 12 hpi. **(a)** Microscopic observation showed that the conidia (c) of *Ctro* germinated to differentiate appressoria (ap), which directly penetrate epidermis to form infection hyphae (ih) in the presence of CEP5p$^{hyp}$. Scale bar = 25 µm. Photos were taken at 12 hpi. Rate of appressoria and infection hyphae formation were calculated as percentages of the number conidia forming appressoria or the number of appressoria forming infection hyphae per total of germinated conidia. The means and SDs were calculated from six independent onion strips with three images taken per strip. Asterisks indicate significant difference among treatments using two-way ANOVA followed by Tukey's multiple comparison test (ns $p > 0.12$). The experiment was repeated twice with similar results and representative results were shown. **(b)** Effect of CEP5p$^{hyp}$ on the growth of *Pta* 6605. CEP5p$^{hyp}$ was added to liquid KB media at 10 or 100 µM (final conc.). The $OD_{600}$ value of *Pta* 6605 cultures at different time points was monitored using Biochrom WPA Colorimeter CO7500 (Biochrom Ltd., Cambridge, UK). The means with SDs were calculated from three replicates in each experiment. Two-way ANOVA followed by Tukey's multiple comparison test was conducted ($p > 0.05$).

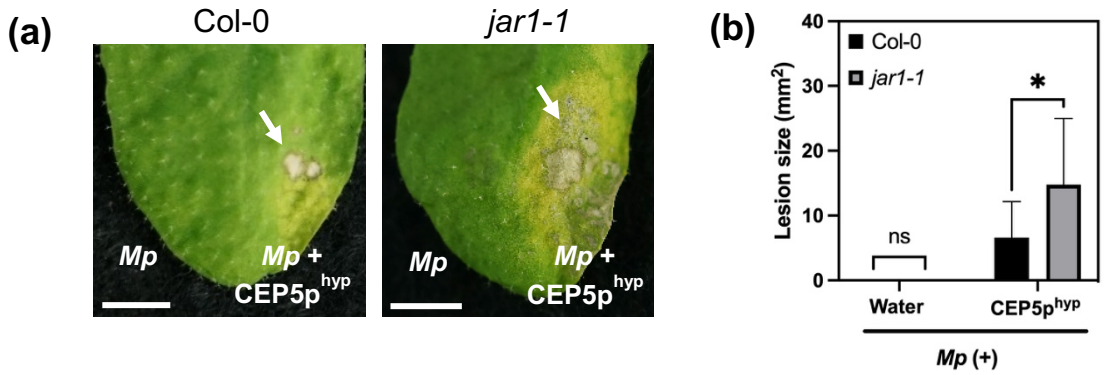



**Fig. S2.2 CEP5p$^{hyp}$ induces susceptibility in *Arabidopsis thaliana* to non-adapted *Mycosphaerella pinodes*. (a)** Detached leaves of Arabidopsis Col-0 and *jar1-1* were drop-inoculated with spore suspension (5 x $10^5$ spores/ml) of *Mycosphaerella pinodes* (*Mp*) strain OMP-1 as described previously (Suzuki at al. 2017) with the presence or absence of synthetic CEP5p$^{hyp}$ (1 mM). Distilled water was used as the control. *Mp* caused more severe symptoms in *jar1-1* mutant plant in the presence of CEP5p$^{hyp}$. Arrows indicate lesions developed on the Arabidosis leaves. Photos were taken at 3 dpi. Scale bar = 1 cm. **(b)** Quantitative analysis of lesion development on Arabidopsis Col-0 and *jar1-1* leaves after inoculated with or without *Mp* spore suspension. Arrows indicate lesions developed on the Arabidosis leaves. The means and SDs were calculated from three independent experiments using Fiji/ImageJ NIH (n=3). Asterisks indicate significant difference among treatments using two-way ANOVA followed by Tukey's multiple comparison test (ns $p > 0.12$; * $p < 0.033$).

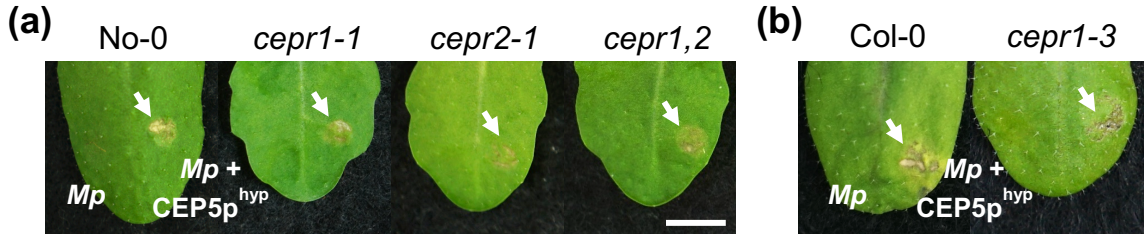



**Fig. S2.3 CEP5p$^{hyp}$ induces susceptibility in Arabidopsis *cepr* mutant plants to another non-adapted fungal pathogen, *Mycosphaerella pinodes* (*Mp*). (a)** Detached leaves of 5 weeks old Arabidopsis No-0, *cepr1-1*, *cepr1-2* and *cepr1,2* was inoculated with spore suspension of *Mp* (5 x $10^5$ spore/ml) in the presence of CEP5p$^{hyp}$ or water as control treatment. **(b)** Detached mature leaves of Col-0 and *cepr1-3* were inoculated with *Mp* spore suspension in the presence of CEP5p$^{hyp}$ or water. (Scale bar = 1 cm).

# Chapter 3: CEP5 peptide negatively modulates defense responses in *Arabidopsis thaliana*


## Abstract

C-terminally encoded peptide (CEP) peptides (CEPs) are known as post-translationally modified peptides consisting of 15 amino acids with one or more hydroxylated proline (hyp) residues. Previously we identified a peptidic fragment from Arabidopsis plants and it is associated with CEP5 peptide (CEP5p). So far, CEP5p has been shown to act through CEP receptors (CEPRs) to control translocation signal for nitrate acquisition and lateral root initiation. Here, we report that CEP5p suppresses Arabidopsis defense responses when induced by bacterial peptide flg22. Arabidopsis plants pretreated with CEP5p also show enhanced disease susceptibility to *Pseudomonas syringae* pv *tomato* DC3000 (*Pto* DC3000), *Pto* DC3000 *hrcC*$^{-}$, and also *Pto* DC3000 *avrRpm1*. Interestingly, CEP5-mediated defense suppression was also observed on *cepr1* and *cepr2* mutant plants, which lack known receptors for nitrate acquisition, suggesting a role of unidentified receptor(s) other than CEPR1 and CEPR2.



**Keywords:** *Arabidopsis thaliana*, CEP, CEPR, *Pseudomonas syringae*, defense suppression

## 3.1 Introduction

Pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) is known as the early defense response in plants against pathogen attack or injury (Bigeard et al. 2015). PTI mostly symptomless, however in the molecular level extensive changes take place to prevent pathogens proliferation in the plant tissues (Szatmári et al. 2014). It is including activation of reactive oxygen species (ROS), callose depositions and activation of defense-related genes (Hatsugai et al. 2017).

In response to plant PTI, adapted pathogens may release effectors, the proteins which produced by plant pathogens to arrest plants defense and allow the pathogens proliferate in the plant tissues. Therefore, pathogens effector recognition by the plant is essential to trigger effector-triggered immunity (ETI). ETI is known as intracellular defense responses that activated after pathogen effectors perception by nucleotide binding-leucine rich repeat (NB- LRR) protein (Toruño et al. 2016). Furthermore, strong immune responses, including program cell death (PCD), can occur at the sites where the pathogens infected the plants which called as the hypersensitive response (HR) (Hatsugai et al. 2017).

From our previous study, we identified a peptidic fragment from Arabidopsis Columbia-0 (Col-0) that can suppress elicitor-induced defense responses, which is tentatively called Arabidopsis endogenous suppressor (ES) (Mai et al. 2020). The partially purified ES preparation severely inhibited PAMP-induced defense responses, i.e., ROS production and expression of defense-related genes (Mai et al. 2020). Based on LC MS and LC MS/MS analyses, C-terminally encoded peptide 5 (CEP5) peptide is identified as one of the possible candidates of Arabidopsis ES.

C-terminally encoded peptide (CEP) peptides were discovered relatively recently using *in silico* approach and characterized as a conserved 15-amino acid peptide domain at or near the C-terminus (Ohyama et al. 2008; Delay et al. 2013; Eves-Van Den Akker et al. 2016). The CEP family in Arabidopsis consist of 15 members with one or more CEP domains and known to be generated from small protein through post-translational modification (Delay et al. 2019). The mature CEPs usually comprise 15 amino acids with one or more hydroxylated proline (hyp) residues (Roberts et al. 2013; Delay et al. 2019).

CEP peptides are known as a root-to-shoot mobile peptide hormones responsible for compensatory nitrogen acquisition in Arabidopsis (Tabata et al. 2014; Ohkubo et al. 2017). Exogenous application of CEP5 peptide is also known to suppress primary and lateral root growth in Arabidopsis (Delay et al. 2013; Roberts et al. 2016). CEPs have been shown to act through two Arabidopsis LRR-RKs later called as CEP receptor 1 and 2 (CEPR1 and 2) during nitrogen demand

signaling (Tabata et al. 2014; Taleski et al. 2018; Chapman et al. 2019). However, CEP peptides function in Arabidopsis defense responses is still unclear.

In our present study, we synthesized CEP5 peptide with hyp residues (CEP5p$^{hyp}$) and showed CEP5p$^{hyp}$ negatively modulates defense responses in Arabidopsis. Furthermore, we explored the involvement of CEPR1 and 2 in suppression of defense responses and could show that CEP5-mediated defense suppression was also found on mutant plants lacking CEPR1 and/or CEPR2.

## 3.2 Materials and methods

### 3.2.1 Plant materials

*Arabidopsis thaliana* ecotype Columbia-0 (Col-0) and Nossen-0 (No-0) were used as wild-type plants in this study. The *cepr1-1*, *cepr2-1, cepr1,2* mutants (No-0) and the *cepr1-3* mutant (Col-0) were obtained from RIKEN Bioresource Research Center Tsukuba, Japan (Tabata et al. 2014). The homozygous *cepr* mutant lines were selected by PCR with loci-specific primers in combination with T-DNA border primer (BP) (**Fig. S3a-c** and **S4a-c**; **Table S3.2**). The Col-0 salicylic acid (SA)-deficient *npr1-2* (Cao et al. 1997) was used to evaluate the effect of CEP5 peptide for induction of susceptibility. Transgenic Col-0 possessing the flg22-induced receptor-like 1 (*FRK1*) promoter with a GUS reporter (*FRK1::GUS*) was also used for *GUS* expression analyses.

### 3.2.2 Plant growth conditions

For pathogen infection assays, ROS generation measurements and callose quantification, Arabidopsis seeds were sown on wetted Jiffy-7 peat pellets (AS Jiffy Products, Oslo, Norway) in the growth chamber for 2 weeks. Germinated seeds were transplanted into soil mixed with vermiculite (1:1) and grown as described by Mai et al. (2020). For MAPK activation, gene expression and GUS expression analyses, Arabidopsis seeds were surface sterilized in 70% ethanol for 10 min, followed by submerging the seeds in 20% (v/v) commercial bleach (6% NaClO) with 0.05% (v/v) of Tween-20 (Bio-Rad, USA) for 15 min with constant shaking. Seeds were washed 7 times with sterile distilled water. Seeds were germinated in 12-well plate containing 2 ml liquid half-strength MS growth medium supplemented with 1% sucrose and 1x Gamborg's vitamin solution (Sigma-Aldrich, St. Louis, MO, USA).

### 3.2.3 Chemicals

Synthetic CEP5$^{hyp}$ (DFR{HYP}TT{HYP}GHS{HYP}GIGH) was synthesized by GL Biochem Ltd. (Shanghai, China) with a purity more than 95%, validated with HPLC and mass spectrophotometry (Roberts et al. 2016). The peptide was suspended in sterile distilled water and used at indicated concentrations in the text and/or figure legends. Synthetic flg22 (Funakoshi, Japan), elf18 and PEP1 were dissolved in sterilized distilled water and used at indicated concentrations. Horseradish Peroxidase (HRP) (Wako, Japan) stock solution (5 mg/mL) was prepared in sterilized distilled water and were used at a final concentration of 10 μg/mL. L-012 probe (Wako, Japan) was dissolved in distilled water as a 20 mM stock solution and was used at a final concentration 200 μM.

### 3.2.4 Pathogen infection assays

For examination of CEP5-mediated disease susceptibility in Col-0, four fully extended leaves of 5-week-old plant were pretreated with water or CEP5$^{hyp}$ peptide (CEP5p$^{hyp}$) at 10 μM final concentration for 3 h and subsequently challenged with *Pseudomonas syringae* pv. *tomato* DC3000 (DC3000), *Pseudomonas syringae* pv. *tomato* DC3000 *hrcC*$^{-}$ (*hrcC*$^{-}$) or *Pseudomonas syringae* pv. DC3000 *avrRpm1* (*avrRpm1*) at a dose $OD_{600}$ = 0.001. The Col-0 *npr1-2* were used as a positive control. The inoculated plants were covered with a clear dome to maintain high humidity and then incubated in a dew chamber for 3 days.

Bacterial growth was assayed 3 days post-inoculation (dpi) by harvesting four infected leaves and two leaf disks were cut from one leaf using a disposable biopsy punch (0.5 cm in diameter; KAI Medical, Gifu, Japan). Eight leaf disks from each plant were divided as two technical repeats and the entire experiment had three biological repeats. The samples were ground using a plastic pestle, diluted serially in sterile distilled water, and plated on King's Broth (KB) agar plates containing nalidixic acid (Nal) with final concentration 10 μg/mL. Bacterial colonies were counted after incubation at $27^{0}$C for 36 h and the colony numbers from selected dilutions were used to calculate colony forming units (CFU).

### 3.2.5 Measurement of ROS generation and detection of $H_2O_2$

Leaf disks from 4–5-week-old plants were excised and then punched using 2 mm diameter biopsy punch. The leaf disks were floated in a petri dish containing filter paper wetted by distilled water and incubated overnight. Prior to elicitation, the elicitation solutions were prepared in three individual wells of a 96-well microtiter plate (Berthold Technologies, Bad Wildbad, Germany)

containing distilled water, HRP, and L-012 probe followed by one of typical PAMPs as a positive control (flg22, elf18 or PEP1) at final concentration 1 µM. Synthetic CEP5p$^{hyp}$ with final concentration ranging from 1000 µM to 10 µM were used and mixed with distilled water, HRP and L-012 probe followed by one of typical PAMPs mentioned above. Final volume for each well was 50 µL, and then 4 leaf disks were carefully floated in each well using precision tweezers. The 96-well microtiter plate was subjected to microplate luminometer reader (TriStar² LB 942, Berthold Technologies, Bad Wildbad, Germany) to measure ROS production between 0 to 3600 seconds.

To detect $H_2O_2$, 14-day-old Col-0 seedlings were soaked in 0.1% (w/v) 3,3′-diaminobenzidine (DAB) (Sigma–Aldrich, St. Louis, MO, USA) solution containing 0.02% (v/v) Tween-20 after treated with water, CEP5p$^{hyp}$ ranging from 10-100 µM, flg22 final concentration 1 µM and the mixture of CEP5p$^{hyp}$ with flg22 following final concentration above for 3 h. The samples then vacuum-infiltrated for 5 min and incubated for 24 h at room temperature. The leaves were decolorized with destaining solutions consisting of ethanol: acetic acid: glycerol (3:1:1 by vol.) at 70$^0$C until the leaves turn translucent. DAB-stained leaves can be visualized as dark brown color.

### 3.2.6 Analysis of MAPK activation

Protein extraction and Western blot analysis were performed as described by Cecile Segonac and Vladimir Nekrasov (2011) with several modifications. 10-day-old seedlings of Arabidopsis Col-0 were treated for 0, 5 or 15 min with water, CEP5p$^{hyp}$ at 100 µM, flg22 final concentration 1 µM and the mixture of CEP5p$^{hyp}$ with flg22 following final concentration above. Treated seedlings were ground and denatured by boiling in 4x sample buffer with ME (Wako, Japan) at 95$^0$C for 3 min. The extracted protein samples were separated by SDS-PAGE and analyzed by Western blot using Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody (Cell Signaling #9101; 1:2,000). MAPK activity was visualized by enhanced chemiluminescence (Thermo Fisher Scientific, Rockford, IL, USA) using a ChemiDocTM Touch Imaging System (Bio-Rad, USA). The intensity of protein bands was quantified using imageJ (http://imagej.nih.gov).

### 3.2.7 Analysis of gene expression by qRT-PCR

For assessing effect of CEP5p on *FRK1* gene expression, 14-day-old Col-0 seedlings were treated with water, flg22 peptide at a final concentration 1 µM, CEP5p$^{hyp}$ ranging from 10-100 µM or a mixture of CEP5p$^{hyp}$ with flg22 with final concentration following final concentration above. Treated seedlings were incubated for 0, 1, 3 and 6 hours at dew chamber at 22$^0$C, with a 10 h light/

14 h dark photoperiod at 11.8 $W.m^{-2}$. Treated seedlings were harvested at indicated time points and subjected to RNA extraction following the isolation kit protocol from Nippon Gene (Tokyo, Japan). The quality of extracted RNA was checked through electrophoresis gel, and the extracted RNA was subjected to reverse transcription PCR to get the cDNA as described previously by Phuong et al. (2020). A qRT-PCR was performed using a Shimadzu GVP-9600 Gene Detection System (Shimadzu, Kyoto, Japan) and the cDNA was amplified using KAPA SYBR FAST universal qPCR master mix (KAPA Biosystems, Boston, MA, USA). The amplification protocol included an initial 3' incubation cycle at $95^0$C for 20 s followed by 40 cycles of $95^0$C 5 s, $60^0$C 20 s, $72^0$C 30 s and a final melt curve analysis. EF1a (*at5g60390*) was used as a control for normalizing the level of cDNA. Relative expression levels of transcripts were calculated using the method of Livak and Schmittgen (2001). The primer sets that we used in this study can be found in **Table S3.1**.

### 3.2.8 GUS staining

To localize the GUS activity, 14-day-old *FRK1::GUS* seedlings were treated with water, $CEP5p^{hyp}$ at 100 µM, flg22 peptide at a final concentration 1 µM and the mixture of $CEP5p^{hyp}$ with flg22 following final concentration above. Treated seedlings were incubated for 2 h at $37^0$C in solution containing 20 mg/ml 5-bromo-4-chloro-3-indolyl-glucuronide (X-Gluc) (Biomedical Science, Japan) in DMF, 50 mM sodium phosphate buffer (pH 7.0) and 7% (v/v) methanol. After incubation, the seedlings were decolorized with 70% (v/v) ethanol and incubated again at $37^0$C overnight. The leaves were photographed using a stereo microscope (Olympus DP70, Tokyo, Japan).

### 3.2.9 Detection of callose deposits

To detect callose deposits, fully expanded 5-week-old Col-0 leaves were syringe-infiltrated with water, flg22 peptide at a final concentration 1 µM, $CEP5p^{hyp}$ at a final concentration 100-10 µM or a mixture of $CEP5p^{hyp}$ with flg22 with final concentration following final concentration above. Syringe-infiltrated leaves were covered with a clear dome to maintain humidity and incubated in a dew chamber at $22^0$C overnight. After incubation, leaves were washed with acetic acid: ethanol (3:1 by vol) followed by ethanol 70% (v/v) for 2 h, and distilled water for 2h. The leaves were stained with 0.5% (w/v) aniline blue (Nacarai Tesque, Tokyo, Japan) in 0.1 M potassium phosphate buffer (pH 9.0) overnight in dark condition, transferred to 50% glycerol (Nacarai Tesque, Tokyo, Japan) and then examined under a fluorescent microscope (Olympus IX70, Tokyo, Japan). Callose deposition area ($um^2$) was calculated using imageJ.

### 3.2.10 Statistical analyses

Statistical analyses were performed using Graphad Prism 9.20 (283) statistic software (Graphad Software, San Diego, CA, U.S.A) with tools for two-tailed unpaired Student's *t* test or two-way analysis of variance (ANOVA) with Tukey's test or Holm-Sidak's test for multiple comparisons of means. Error bars in figures represent standard deviations. The *P*-values of statistical analyses are provided in figure legends. Asterisks (*) are used to show significant statistical differences between two samples.

## 3.3 Results

### 3.3.1 CEP5 peptide induces disease susceptibility in Col-0 against DC3000, *hrcC*$^{-}$ and *avrRpm1*

Synthesized CEP5p$^{hyp}$ was shown to induce susceptibility in Col-0 against non-adapted bacterial pathogen, *Pseudomonas syringae* pv. *tabaci* 6605 (Aprilia et al. in prep.). To test whether PTI in Col-0 is affected by CEP5p$^{hyp}$ treatment, we challenged Col-0 plants with DC3000 and *hrcC*$^{-}$, the avirulent DC3000 strain which is defective in type III secretion system (T3SS) as well as one expressing *avrRpm1* (*avrRpm1*). As shown in **Fig. 3.1a**, infiltration assays with DC3000 revealed that Col-0 plants pretreated with synthetic CEP5 peptide at 10 μM were more susceptible than the plants pretreated with water. Col-0 plants pretreated with CEP5p$^{hyp}$ also developed water-soaked symptoms compared unlike water pretreated plants when inoculated with *hrcC*$^{-}$ as well with *avrRpm1* (**Fig. 3.1a**). However, a synthetic variant with four amino acids substitution while retaining hyp residues at the same position (mCEP5p$^{hyp}$) did not show similar enhanced susceptibility (**Fig. S3.1a-b**).

The SA-deficient mutant *npr1-2* (for *non-expressor of pathogenesis-related genes 1-2*), previously describe to be more susceptible to DC3000 (Shi et al. 2010), was used as a control and assayed against DC3000, *hrcC*$^{-}$ or *avrRpm1*. As occurred with Col-0 pretreated with CEP5p$^{hyp}$, *npr1-2* plants pretreated with water also showed similar symptoms development. The rate growth of DC3000, *hrcC*$^{-}$ and *avrRpm1* in Col-0 pretreated with CEP5 peptide was significantly higher than that measured in water pretreated Col-0 (**Fig. 3.1b**). However, the rate of bacterial growth observed in *npr1-2* plants was comparable between both treatments. Taken together, these observations clearly indicate that exogenous CEP5p$^{hyp}$ can suppress the Arabidopsis immunity via affecting the common pathway(s) or process(es) leading to the PTI and ETI.

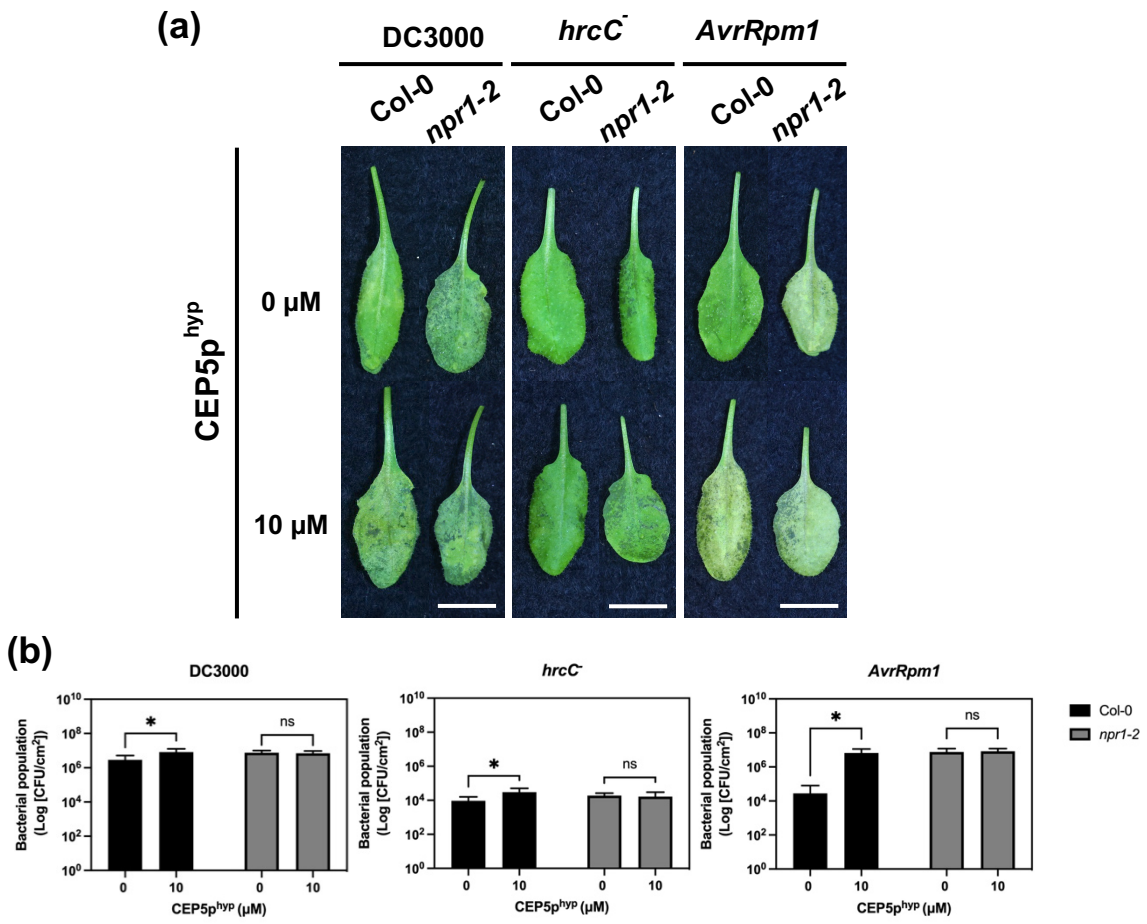



**Fig. 3.1 CEP5p$^{hyp}$ induces susceptibility in Col-0 to DC3000, *hrcC*$^{-}$ and *avrRpm1***

(a) Disease phenotype of syringe-infiltrated Col-0 and *npr1-2* plants at 3 dpi. Leaves of 5-week-old Col-0 and *npr1-2* were syringe-infiltrated with a bacterial suspension of DC3000, *hrcC*$^{-}$ or *avrRpm1* ($OD_{600}$: 0.001) after pretreated with CEP5p$^{hyp}$ at 10 μM for 3 h.

(b) Quantification of bacterial population of DC3000, *hrcC*$^{-}$ or *avrRpm1* at 3 dpi. The means and SDs were calculated from three biological replicates. The experiment was repeated three times, with similar results. Asterisks indicate significant differences from water treatment using two-way ANOVA followed by Holm-Sidak's multiple comparison test (***ns*** $p > 0.12$; ** $p < 0.002$; *** $p < 0.001$).

## 3.3.2 CEP5 peptide suppresses PAMPs- and DAMP-induced ROS generation in Arabidopsis Col-0

Reactive oxygen species (ROS) is the product from reduction of molecular oxygen: superoxide radical ($\cdot O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical (·OH) (Wojtaszek 1997). Activation of ROS at the plant cell surface, well known as oxidative burst, is the earliest cellular defense response that activated by plant to cope with biotic or abiotic stresses (Yoshioka et al. 2008). ROS production

in plant can trigger the activation of further plant defense responses such as synthesis of salicylic acid (SA), activation of MAP Kinases (MAPK) cascades, including activation of systemic acquired resistance (SAR) (Wojtaszek 1997; Yoshioka et al. 2008). However, it has been previously reported that pathogen effectors and pathogen suppressors can suppress oxidative burst to breach plant PTI responses and successfully invade the plant (Hemetsberger et al. 2012; Toyoda et al. 2016).

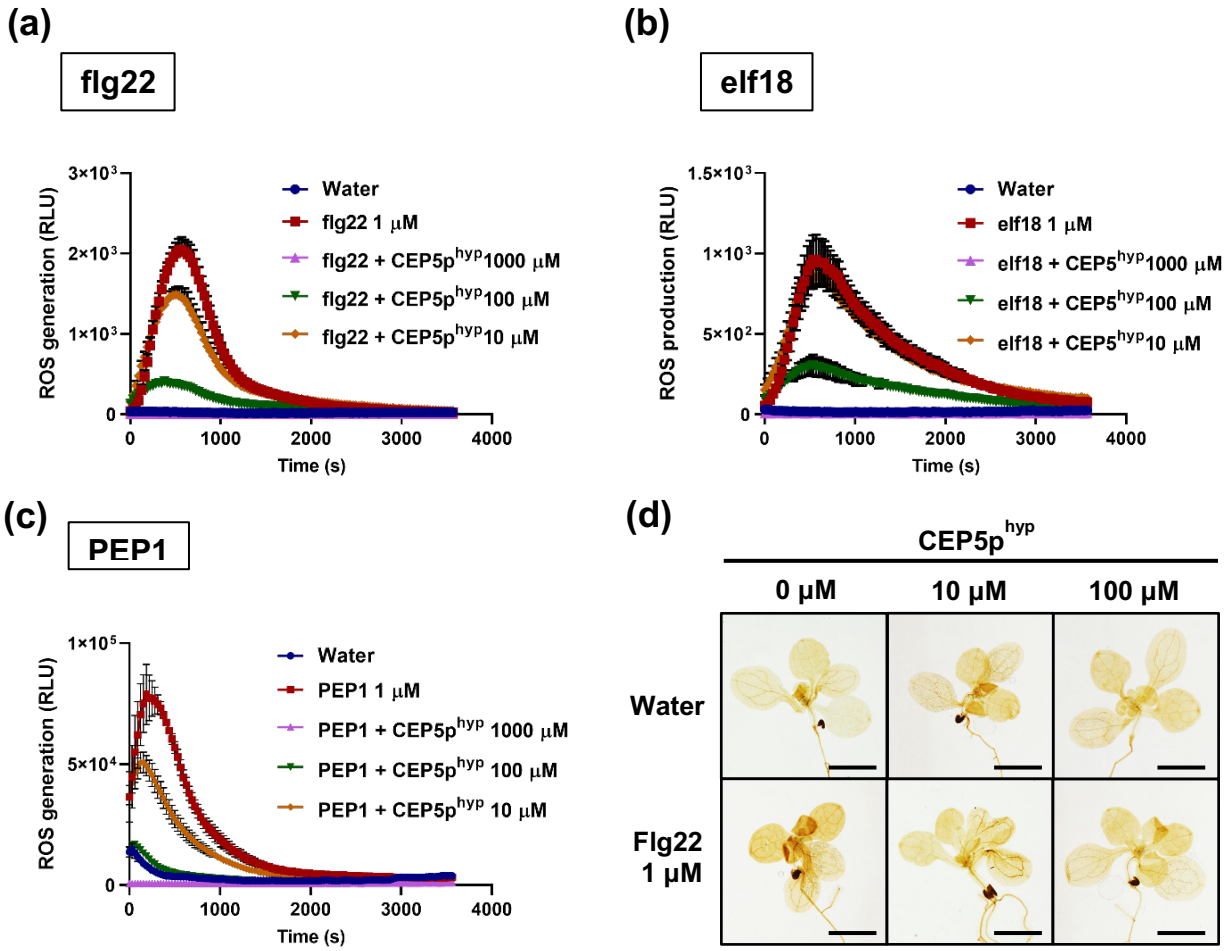



**Fig. 3.2 CEP5p$^{hyp}$ inhibits ROS production and $H_2O_2$ accumulation in Col-0 challenged with typical PAMPs or DAMP**

(a)-(c) Suppression of ROS generation in Col-0 by flg22, elf18 and PEP1 respectively, in the absence or presence of CEP5p$^{hyp}$ on the indicated concentrations. The data represent the means and SEs; $n = 3$ of one biological experiment. The experiment was repeated three times independently with similar results.

(d) CEP5p$^{hyp}$ suppresses $H_2O_2$ accumulation in Arabidopsis induced by flg22. Samples were taken at 3 hpt. The experiment was repeated three times with similar results. Representative results are shown.

Here we revealed CEP5p$^{hyp}$ acts as PAMP-derived suppressor which can suppress typical PAMP-induced ROS generation in Arabidopsis such as: flg22, elf18, and DAMP-induced ROS generation, PEP1 (**Fig. 3.2a-c**). Our results revealed that, at least 100 µM of CEP5p$^{hyp}$ was required to suppress ROS production in Arabidopsis when induced by typical PAMPs or DAMP mentioned above. On the other hand, in the presence of CEP5p$^{hyp}$ at 10-100 µM, the accumulation of $H_2O_2$ when induced by flg22 as typical PAMP also suppressed compared to flg22 treatment without CEP5p$^{hyp}$ (**Fig. 3.2d**). These findings suggest that CEP5p$^{hyp}$ targets common PAMPs signaling pathway in Arabidopsis and in result, directly inhibit Arabidopsis ROS production.

### 3.3.3 CEP5 peptide suppresses MAPK activity in Arabidopsis

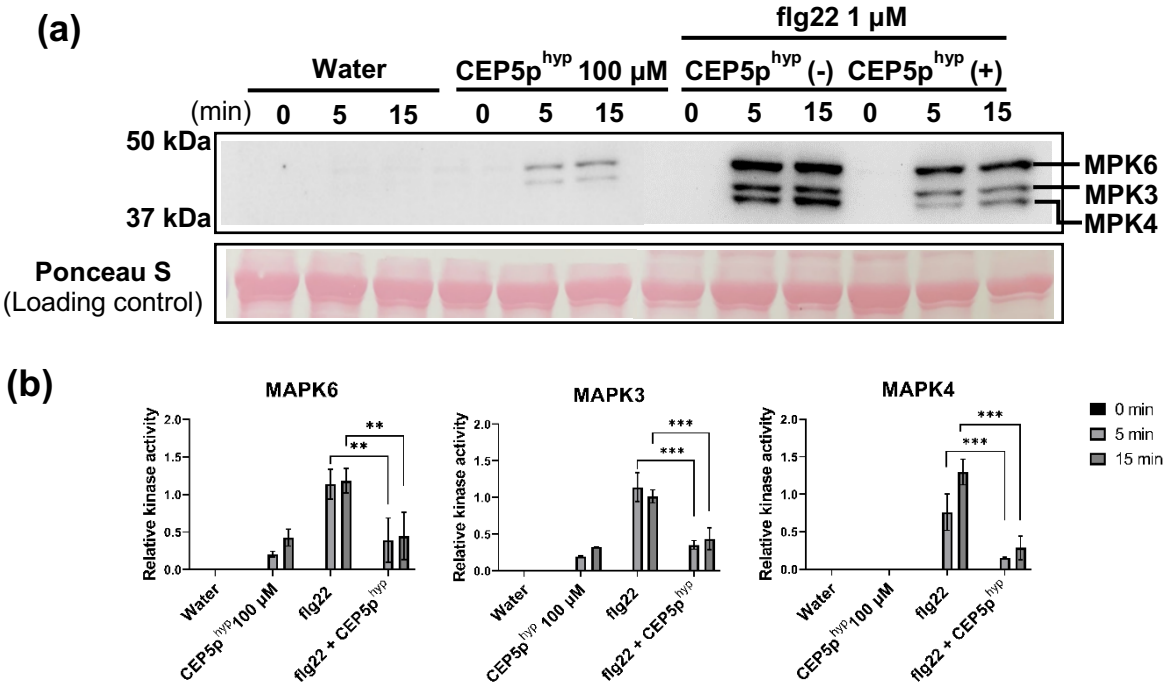



**Fig. 3.3 CEP5p$^{hyp}$ suppresses flg22-induced MAPK activation in Arabidopsis Col-0.**

(a) CEP5p$^{hyp}$ at 100 µM suppresses flg22-induced MAPK activation in Col-0 from 5-15 min post treatment. Activated MAPKs were detected by immunoblots using Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody. Equal loading of protein samples is indicated by the Ponceau S staining of Rubisco.

(b) Quantification of the relative intensity of MAPK6, MAPK3 and MAPK4 bands in Col-0 after flg22 and/or CEP5p$^{hyp}$ treatment. The means and SDs were calculated from two independent experiments. Asterisks indicate significant differences from flg22 treatment as the positive control using two-way ANOVA followed by Tukey's multiple comparison test (** $p < 0.002$; *** $p < 0.001$).

MAP kinases (MAPKs) cascade, play an important role during plant growth and development, including signaling responses for biotic or abiotic stresses (Meng & Zhang 2013). Activation of MAPK mediated by upstream MAPKKK (MAP Kinase Kinase Kinase), MAPKK (MAP Kinase Kinase) and a MAPK via phosphorelay mechanisms to downstream targets (Pitzschke et al. 2009; Meng & Zhang 2013). MAPK 3 and MAPK 6 are responsible for biotic and abiotic stresses and play an important role during plant growth and development. On the other hand, MAPK4 and MAPK11 responsible during pathogen defense responses and in response to abiotic stress (Meng & Zhang 2013).

Here we demonstrated that in the presence of $CEP5^{hyp}$ peptide at 100 µM, MAPK cascade was suppressed or delayed when induced by flg22 at 1 µM (**Fig. 3.3a**). MAPK cascade was suppressed by $CEP5^{hyp}$ peptide from 5 to 15 minutes post-treatment. We also tried to quantify MAPK activity using imageJ and the activity of MAPK3, MAPK6, and MAPK4 were suppressed or delayed in the presence of $CEP5^{hyp}$ peptide from 5 to 15 minutes post-treatment compared to flg22 treatment (**Fig. 3.3b**). These results suggest that as CEP5 peptide suppressed ROS generation by targeting common PAMPs signaling pathway, the signal transduction to activate MAPK cascade also inhibited by CEP5 peptide and resulting suppression or delay the MAPK activity when induced by typical PAMP, flg22.

### 3.3.4 CEP5 peptide suppresses expression of *FRK1* gene in Arabidopsis

Flg22–induced receptor-like kinase 1 (*FRK1*) is common defense-related gene marker in Arabidopsis (Yeh et al. 2015). Our result revealed that, in the presence of $CEP5^{hyp}$ peptide from10-100 µM, the expression of *FRK1* were suppressed significantly from 1 to 6 hours post treatment (**Fig. 3.4a**). These results suggest that following suppression of MAPK activity, subsequent expression of *FRK1* gene were also suppressed or delayed in the presence of $CEP5p^{hyp}$. Therefore, $CEP5p^{hyp}$ can inhibit Arabidopsis upstream and downstream responses such as MAPK activity and expression of *FRK1* gene.

To confirm the effect of $CEP5p^{hyp}$ treatment in the expression level of *FRK1* gene, transgenic Col-0 possessing the *FRK1* promoter with a GUS reporter was used. Histochemical GUS staining revealed that *FRK1* was highly expressed in the seedlings treated with 1 µM flg22 (**Fig. 3.4b**). However, in the presence of $CEP5p^{hyp}$ at 100 µM, the expression level of FRK1 was significantly reduced supporting gene expression results.

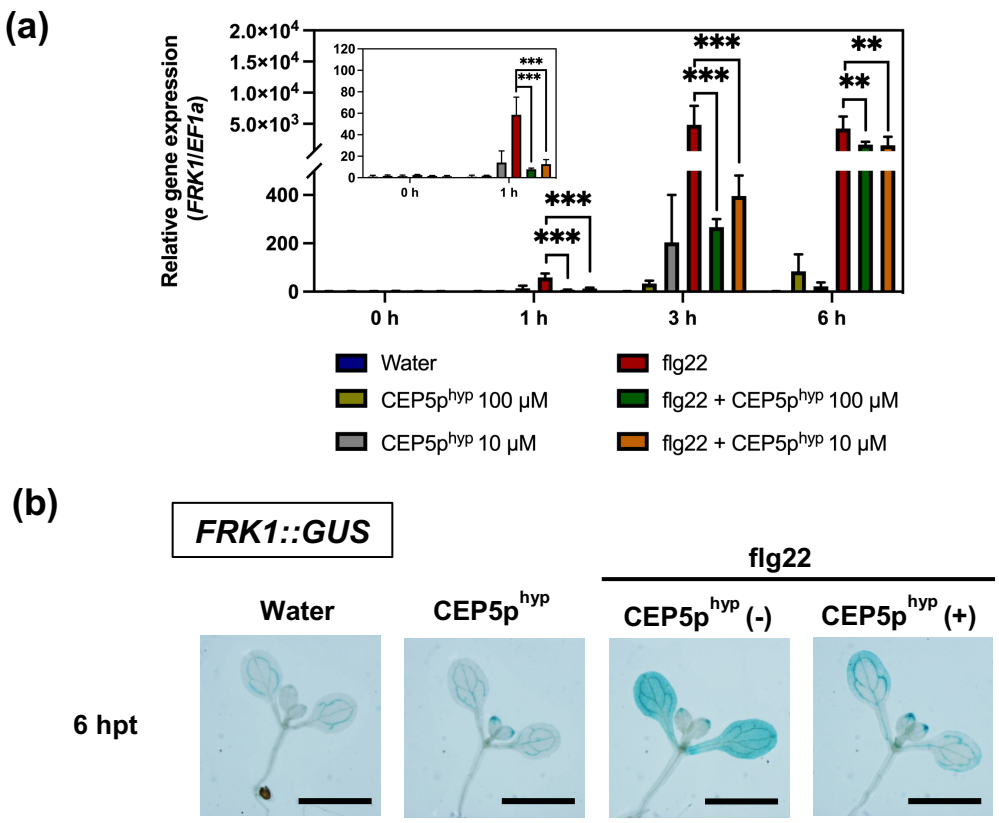



**Fig. 3.4 CEP5p$^{hyp}$ suppresses flg22-induced *FRK1* gene in Arabidopsis Col-0.**

(a) Quantitative RT-PCR analysis of flg22-induced *FRK1* gene expression after treated with flg22 and/or CEP5p$^{hyp}$. Col-0 seedlings were collected at 0-6 h after treatment. Values were normalized to the level of *EF1a*. Error bars represent means and SDs of three reactions in each experiment. The experiment was repeated three times with similar results; representative results are shown. Asterisks indicate significant difference among treatments using two-way ANOVA with Tukey's multiple comparison test (** $p < 0.002$; *** $p < 0.001$).

(b) Histochemical GUS staining of *FRK1::GUS*. Representative images of Arabidopsis seedlings treated with flg22 at 1 μM and/or CEP5p$^{hyp}$ at 100 μM are shown. Bars = 2 mm.

### 3.3.5 CEP5 peptide suppresses callose deposition in Arabidopsis

Plants defend themselves from pathogens attack by following mechanisms: (1) hypersensitive response (HR), (2) phytoalexin production such as camalexin in Arabidopsis, (3) biosynthesis degradative enzyme such as chitinase for degrading fungal cell wall, and (4) cell wall modification by thickening the cell wall with callose deposits in the infection sites (Voigt & Somerville 2009). Here our result showed that in the presence of CEP5$^{hyp}$ peptide at 10-100 μM, callose deposition in Arabidopsis was suppressed when induced by typical PAMP, flg22 at 1 μM (**Fig. 3.5a**).

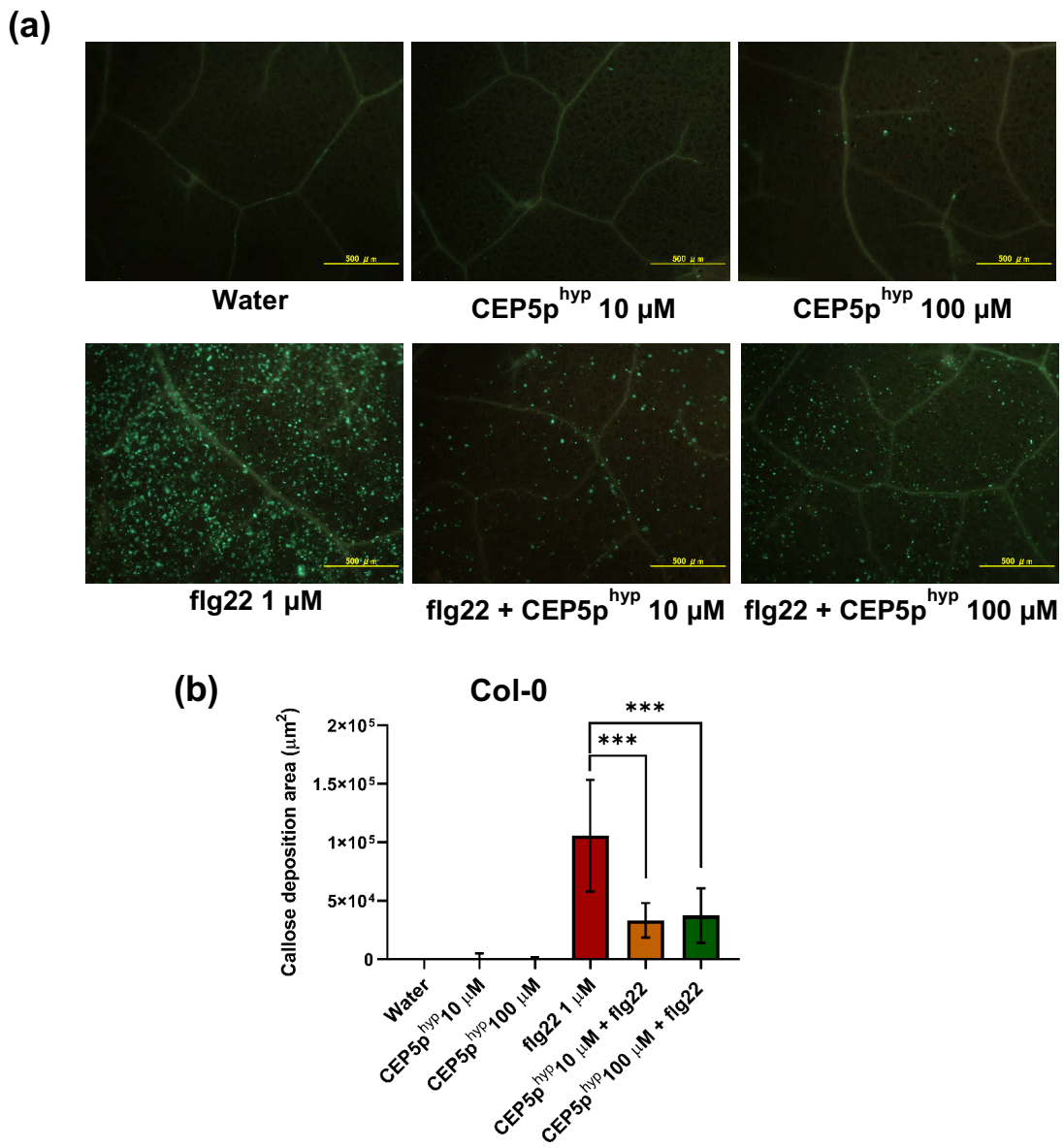



**Fig. 3.5 CEP5p$^{hyp}$ suppresses callose deposition in Col-0.**

(a) flg22-induced Callose deposition in Col-0 leaves in the presence or absence CEP5p$^{hyp}$ at 10-100 μM. The experiment was repeated three times with similar results. Bars = 500 μm.

(b) Quantification of callose deposition area after flg22 and/or CEP5p$^{hyp}$ treatment. Error bars represent means and SDs of three technical repeats and the entire experiment had three biological repeats ($n$ = 9). The experiment was repeated three times with similar results.

Interestingly, CEP5$^{hyp}$ peptide treatment without flg22 slightly induced callose deposition in Arabidopsis. We assumed because of Arabidopsis perceive CEP5p$^{hyp}$ as unknown compound. We also tried to quantify callose deposits using imageJ and the graphic showed that compared to flg22

treatment, in the presence of CEP5p$^{hyp}$, callose deposits in Arabidopsis were significantly suppressed (**Fig. 3.5b**). This finding suggests that CEP5p$^{hyp}$ possibly suppresses defense responses in Arabidopsis starting from ROS generation, MAPK activity, including callose deposition which lead to successful infection by pathogens.

### 3.3.6 Loss of CEPR1 and CEPR2 does not diminish the suppression of defense responses by CEP5 peptide

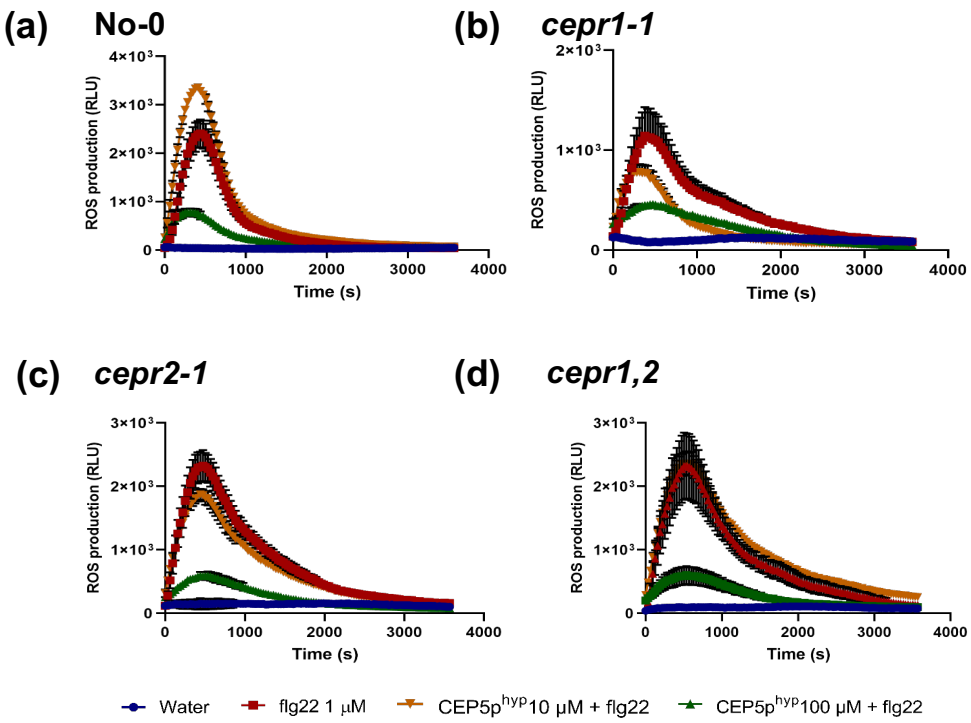



**Fig. 3.6 CEP5p$^{hyp}$ suppresses flg22-induced ROS production in No-0 and *cepr* mutants.**

(a)-(d) flg22-induced ROS generation was suppressed in the absence or presence of CEP5p$^{hyp}$ at 10-100 μM in No-0 and *cepr* mutant plants. The data represent the means and SEs; $n$ = 3 of one biological experiment. The experiment was repeated three times independently with similar results.

CEPR1 and CEPR2 have been identified as shoot-expressed receptors for CEP peptide (Ohkubo et al. 2017). From the above results, we hypothesized the loss of CEP receptors will diminish CEP5-mediated suppression in response to flg22. To test this, we examined effect of CEP5p$^{hyp}$ in ROS generation in No-0 (WT) and *cepr* mutant plants induced by flg22. Flg22 treatment resulted in higher ROS generation in WT and *cepr* mutant plants compared with water treatment (**Fig. 3.6a-d**). However, in the presence of CEP5p$^{hyp}$ at 100 μM, ROS generation in WT and *cepr* mutant plants were

significantly suppressed (**Fig. 3.6a-d**). Unlike 100 μM of CEP5p$^{hyp}$ treatment, in the presence of CEP5p$^{hyp}$ at 10 μM, the ROS generation in WT and *cepr1-1 cepr2-1* was higher than flg22 treatment (**Fig. 3.5a and d**), and slightly suppressed in *cepr1-1* and *cepr2-1* (**Fig. 3.6b and c**).

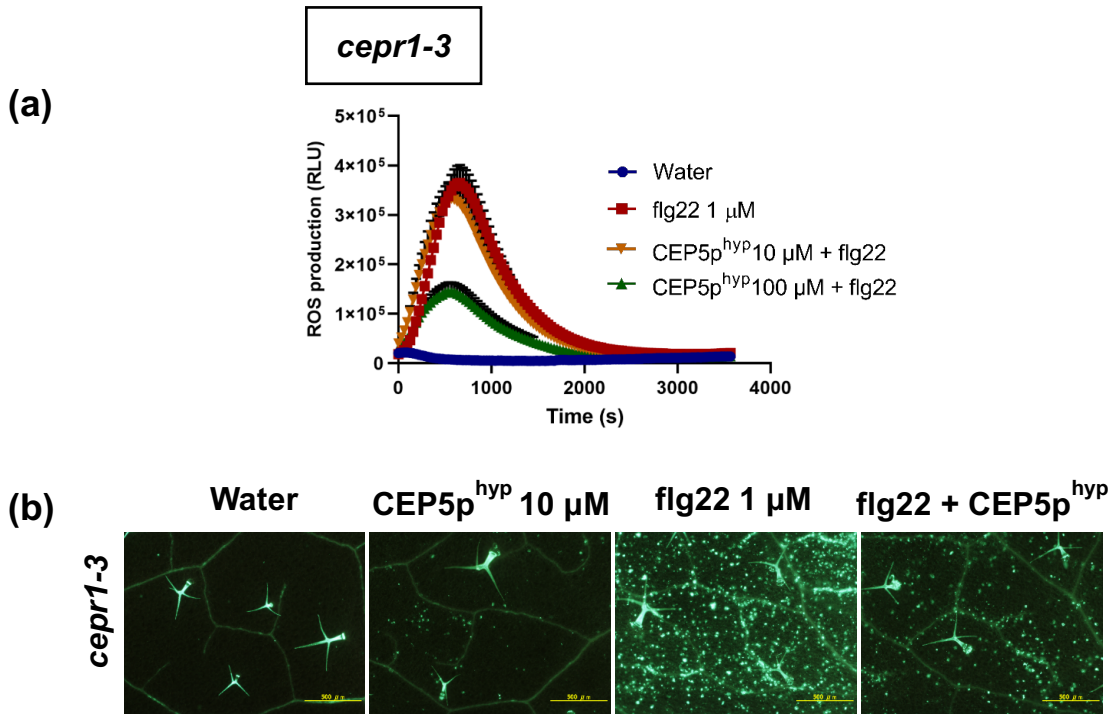



**Fig. 3.7 CEP5p$^{hyp}$ suppresses flg22-induced ROS production and callose deposition in *cepr1-3* mutant plant.**

(a) flg22-induced ROS generation was suppressed in the absence or presence of CEP5p$^{hyp}$ at 10-100 μM in *cepr1-3* mutant plant. The data represent the means and SEs; $n$ = 3 of one biological experiment. The experiment was repeated three times independently with similar results.

(b) Similar to other *cepr* mutant plants, callose deposition in *cepr1-3* was also suppressed in the presence of CEP5p$^{hyp}$.

The CEP5-mediated defense suppression was also found on an additional CEPR1 knockout allele, *cepr1-3* from a Col-0 background. Flg22-induced ROS generation in *cepr1-3* was suppressed in the presence of CEP5p$^{hyp}$ at 100 μM, but not 10 μM, compared with flg22 treatment (**Fig. 3.7a**). However, flg22-induced callose deposition assay revealed that pretreated *cepr1-3* with CEP5p$^{hyp}$ at 10 μM decrease callose deposits in response to flg22 (**Fig. 3.7b**). Taken together, these data suggest that CEP known receptors, CEPR1 and 2, most likely do not contribute to the activation of CEP5-mediated defense suppression by PAMPs or pathogens.

## 3.4 Discussions

CEP peptides have been shown to act through CEP receptors (CEPRs) to control translocation signal for nitrate acquisition and lateral root initiation (Delay et al. 2013; Roberts et al. 2013; Tabata et al. 2014; Ohkubo et al. 2017), but little is known whether and to what degree CEPs regulate plant defense responses. Here, we provide evidence that one of CEP peptides, CEP5, act as defense suppressor when applied exogenously.

In this study, we demonstrate that pretreated Col-0 plants with CEP5p$^{hyp}$ show enhanced disease susceptibility to virulent bacterium DC3000 and to the avirulent *hrcC*$^{-}$ as well as one expressing *avrRpm1*. This enhanced susceptibility almost similar with *npr1-2* plants pretreated with water. However, pretreated *npr1-2* plants with CEP5p$^{hyp}$ does not show enhanced disease susceptibility to all bacterial pathogens tested. To our knowledge, Arabidopsis resistance to DC3000 depends on salicylic acid (SA), and mutants compromised in SA biosynthesis (e.g., *sid2-1*) or perception (e.g., *npr1-2*) are more susceptible to DC3000 (Agorio & Vera 2007). The fact that pretreatment by CEP5p$^{hyp}$ causes enhanced susceptibility in Col-0, suggesting that a compromised perception of SA in Col-0 because of CEP5p$^{hyp}$ pretreatment similar in *npr1-2*. In addition, in the presence of CEP5p$^{hyp}$, the expression level of *PR1* gene significantly suppressed when induced by exogenous SA (**Fig. S3.2a**). The expression level of *PR1* gene in transgenic Col-0 possessing the *PR1* promoter with a GUS reporter (*PR1::GUS*) also support gene expression analysis results (**Fig. S3.2b**), suggesting that CEP5p$^{hyp}$ most likely disrupts SA signaling, resulting in the suppression of *PR1* gene expression level and contributes to enhanced disease susceptibility in Col-0.

Upon PAMPs recognition, plants can induce PTI responses to limit invading pathogens, including generation of ROS, activation of MAPKs, and production of defense-related proteins/peptides (Boller & Felix 2009; Bigeard et al. 2015). Plants are also able to detect DAMPs and perception of DAMPs also triggers immune responses, known as DAMP-triggered immunity, with overlapping signaling networks (Hou et al. 2019). Here, we have found that CEP5p$^{hyp}$ acts as PAMPs/DAMP-derived suppressor which can suppress typical PAMPs/DAMP-induced ROS generation, flg22, elf18 and PEP1, in Col-0. These results suggest that CEP5p$^{hyp}$ has robust inhibitory effect by targeting common PAMPs and DAMP signaling pathway.

MAP kinases (MAPKs) cascade is activated downstream of PAMP receptors and involved in transducing defense signals leading to expression of defense related genes (Tsuda et al. 2013; Sun et al. 2018). CEP5p$^{hyp}$ reduced flg22-induced MAPK activation from 5 to 15 min post treatment,

likewise flg22-induced *FRK1* gene expression from 1-6 hpt. Consistent with the involvement of CEP5p$^{hyp}$ in the inhibition of flg22-induced *FRK1* gene expression, *FRK1* expression in transgenic Col-0, *FRK1::GUS*, also reduced significantly. These data suggest that CEP5p$^{hyp}$ most likely compromised upstream and downstream defense signals upon PAMPs perception.

PAMP-induced callose deposition classified as late PTI response, it is because callose deposits normally can be observed hours after pathogen attack (Ellinger & Voigt 2014). PAMPs perception results in callose deposition at the cell wall and can be suppressed by pathogen effectors targeting PTI responses (Zhang et al. 2007; Ellinger & Voigt 2014). Here, flg22-induced callose deposition significantly suppressed in Col-0 pretreated with CEP5p$^{hyp}$ from 10-100 μM, thus implying that similar to pathogen effectors, CEP5p$^{hyp}$ targets early PTI responses resulting in the suppression of callose deposition as late PTI response.

CEP-CEPR interactions mainly focused on CEP peptides production in roots responding to low N in the root ecosystem (Chapman et al. 2019). CEPR1 knockout allele from No-0 and Col-0 background, *cepr1-1* and *cepr1-3*, show longer primary root than wild-type when grown on agar medium supplemented with CEP peptide (Tabata et al. 2014; Chapman et al. 2019). Interestingly, in this study CEP5-mediated defense suppression was also observed on *cepr1* and *cepr2* mutants, which lack known receptors for nitrate acquisition, CEPR1 and CEPR2 (**Fig. 3.6a-d**; **3.7a-b**; **Fig. S3.3a-b**). Hence, there should be a role of unidentified receptor(s) other than CEPR1 and CEPR2 that contributes to the suppression of PTI responses in Arabidopsis.

## 3.5 Supplementary information

**Table S3.1** Primer sequences used for qRT-PCR

| Gene | Accession | Sequence (5' > 3') | References |
|---|---|---|---|
| ***EF1a*** | *At5g60390* | F : CATCATTTGGCACCCTTCTT<br>R : TGGTGACGCTGGTATGGTTA | Phuong et al. 2020 |
| ***FRK1*** | *At2g19190* | F : ACGGGCATAGTTCCACAAAG<br>R : CGTCAAAAGAACGACGATGA | Yeh et al. 2015 |
| ***PR1*** | *At2g14610* | F : CCCACAAGATTATCTAAGGGTT<br>R : CCCTCTCGTCCCACTGCAT | Phuong et al. 2020 |

**Table S3.2** Primer sequences used for *genotyping cepr* mutant plants

| Primer name | Sequence (5' > 3') | References |
|---|---|---|
| ***5-1a*** | CATCATTTGGCACCCTTCTT | |
| ***3-1a*** | ACGGGCATAGTTCCACAAAG | |
| ***cepr1-1*** | A : AGTCCGCAACTTTAGGCTGA<br>B: ATTCCACCAACTGCTGGTTC | Tabata et al. 2014;<br>Kuromori et al. 2004 |
| ***cepr2-1*** | A: GGAAAACTCGTTCTGCAAGG<br>B: TCCCGCCAGAGATAGTCAAC | |
| ***cepr1-3 (RP)*** | AATTGGGAGAATTAAAACCGG | |
| ***cepr1-3 (LP)*** | TGAGTGGCGTACTCGTCATC | |
| ***cepr1-3 (BP)*** | ATATTGACCATCATACTCATTGC | Chapman et al. 2019 |
| ***CEPR1 (At5g49660)*** | F: CTTGTGGACAAGAACATCGTAGG<br>R: GATCAGAAGCTGAACAACTTCGTT | |

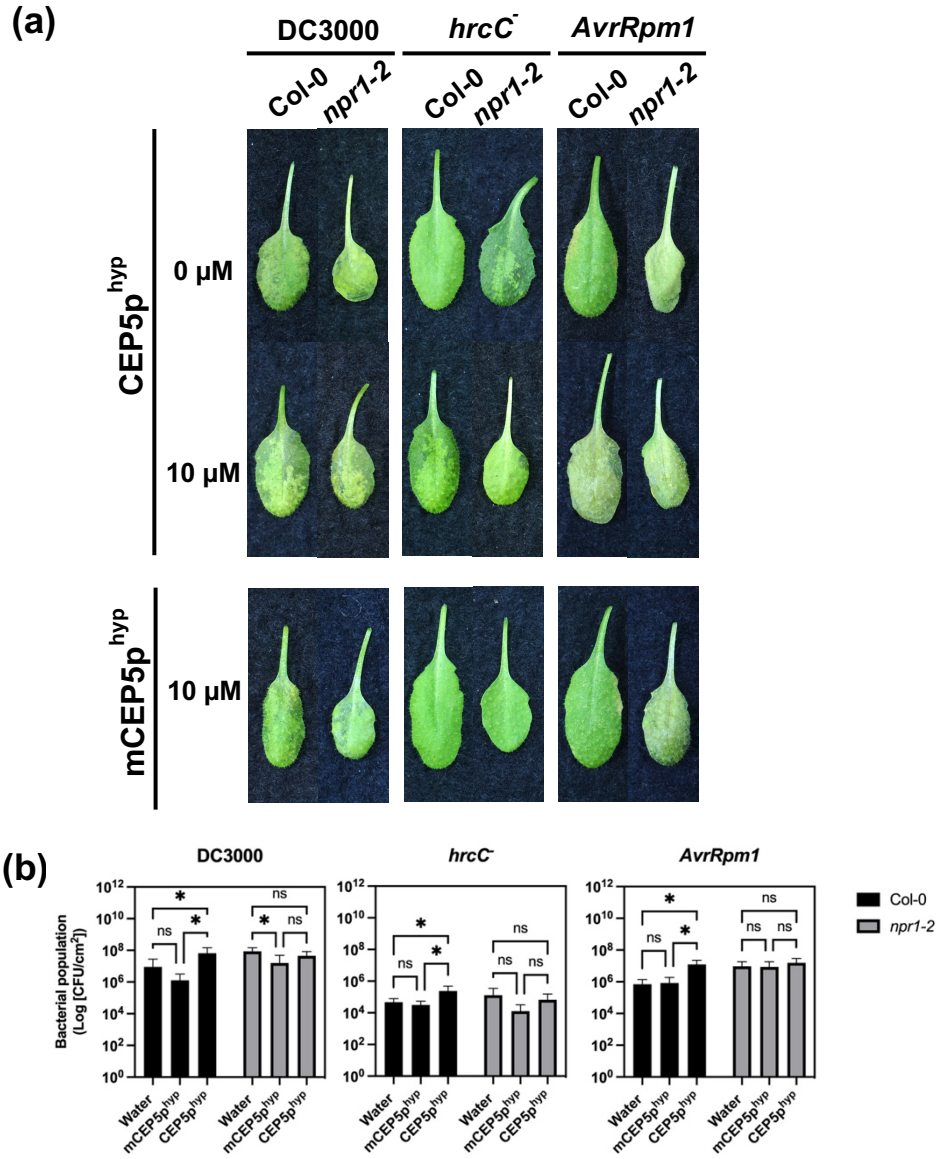



**Fig. S3.1 mCEP5p$^{hyp}$ cannot induce susceptibility in Col-0 to DC3000, *hrcC*$^{-}$ and *avrRpm1***

(a) Disease phenotype of syringe-infiltrated Col-0 and *npr1-2* plants at 3 dpi. Leaves of 5-week-old Col-0 and *npr1-2* were syringe-infiltrated with a bacterial suspension of DC3000, *hrcC*$^{-}$ or *avrRpm1* ($OD_{600}$: 0.001) after pretreated with distilled water, CEP5p$^{hyp}$ or mCEP5$^{hyp}$ at 10 µM for 3 h.

(b) Quantification of bacterial population of DC3000 , *hrcC*$^{-}$ or *avrRpm1* at 3 dpi. The means and SDs were calculated from three biological replicates. The experiment was repeated two times, with similar results. Asterisks indicate significant differences from water treatment using two-way ANOVA followed by Tukey's multiple comparison test (***ns*** $p > 0.12$; ***** $p < 0.033$).

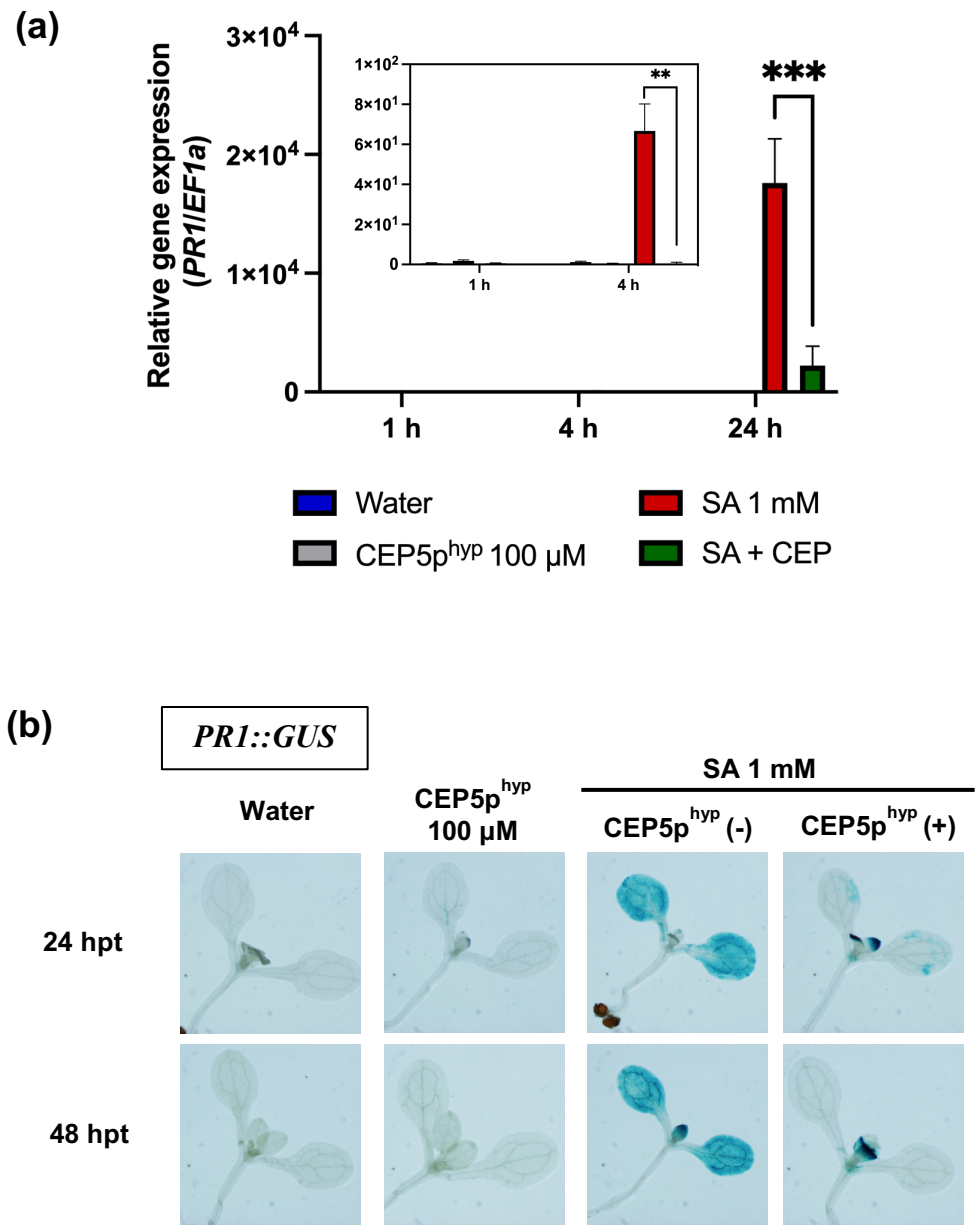



**Fig. S3.2 CEP5p$^{hyp}$ suppresses SA-induced *PR1* gene expression.**

(a) Quantitative RT-PCR analysis of SA-induced *PR1* gene expression after treated with exogenous SA and/or CEP5p$^{hyp}$ at 100 μM. Col-0 seedlings were collected at 1, 4 and 24 h after treatment. Values were normalized to the level of *EF1a*. Error bars represent means and SDs of three reactions in each experiment. The experiment was repeated three times with similar results; representative results are shown. Asterisks indicate significant difference among treatments using two-way ANOVA with Tukey's multiple comparison test (** $p < 0.002$; *** $p < 0.001$).

(b) Histochemical GUS staining of *PR1::GUS*. Representative images of Arabidopsis seedlings treated with exogenous SA and/or CEP5p$^{hyp}$ are shown. Samples were taken at 24 and 48 hpt and the experiment was repeated three times. Bars = 2 mm.

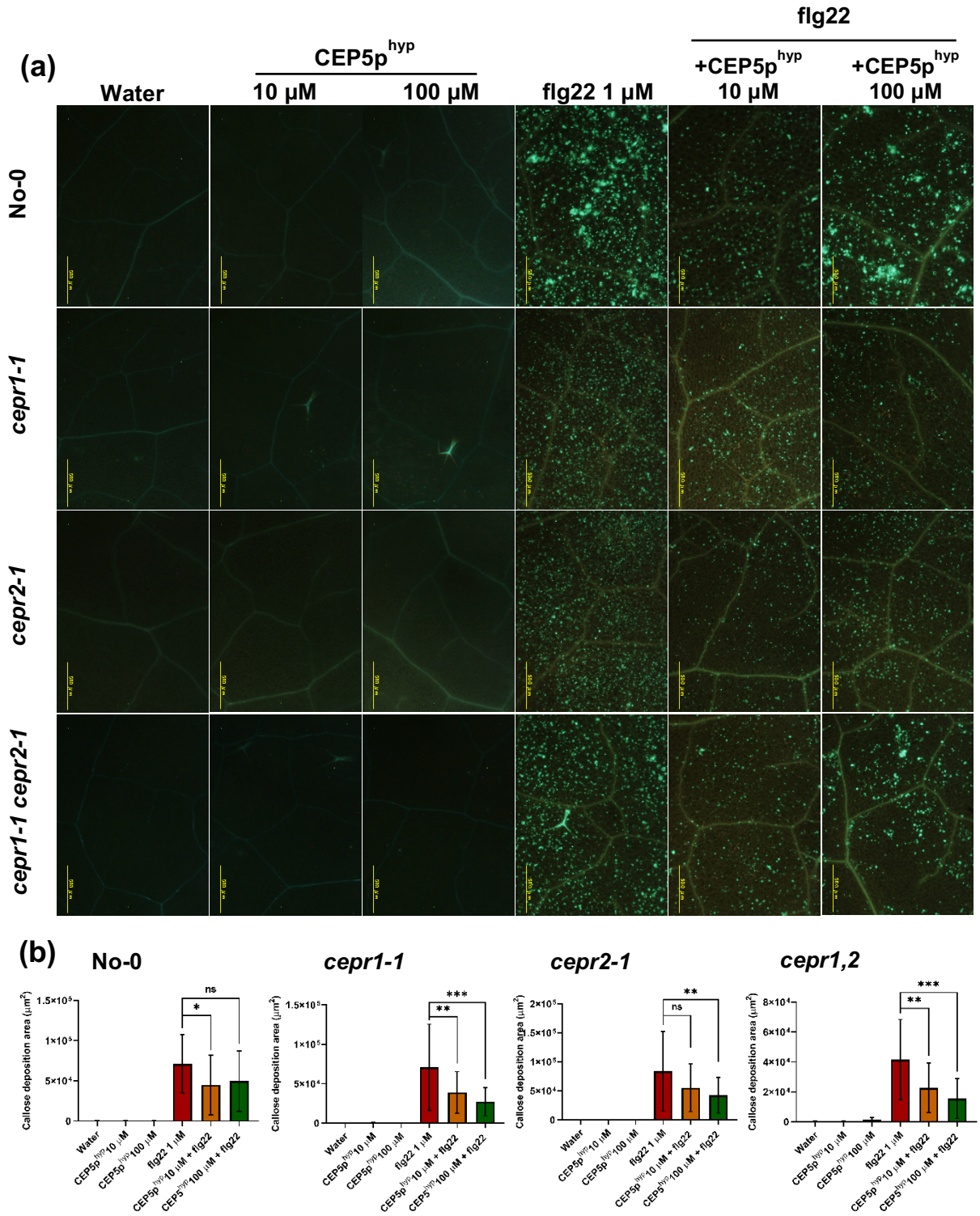



**Fig. S3.3 CEP5p$^{hyp}$ suppresses flg22-induced callose deposition in No-0 and *cepr* mutant plants.**

(a) flg22-induced Callose deposition in No-0 and *cepr* mutant plants leaves in the presence or absence CEP5p$^{hyp}$ at 10-100 μM. The experiment was repeated two times with similar results. Bars = 500 μm.

(b) Quantification of callose deposition area after flg22 and/or CEP5p$^{hyp}$ treatment. Error bars represent means and SDs of three technical repeats and the entire experiment had three biological repeats (n = 9). The experiment was repeated two times with similar results.

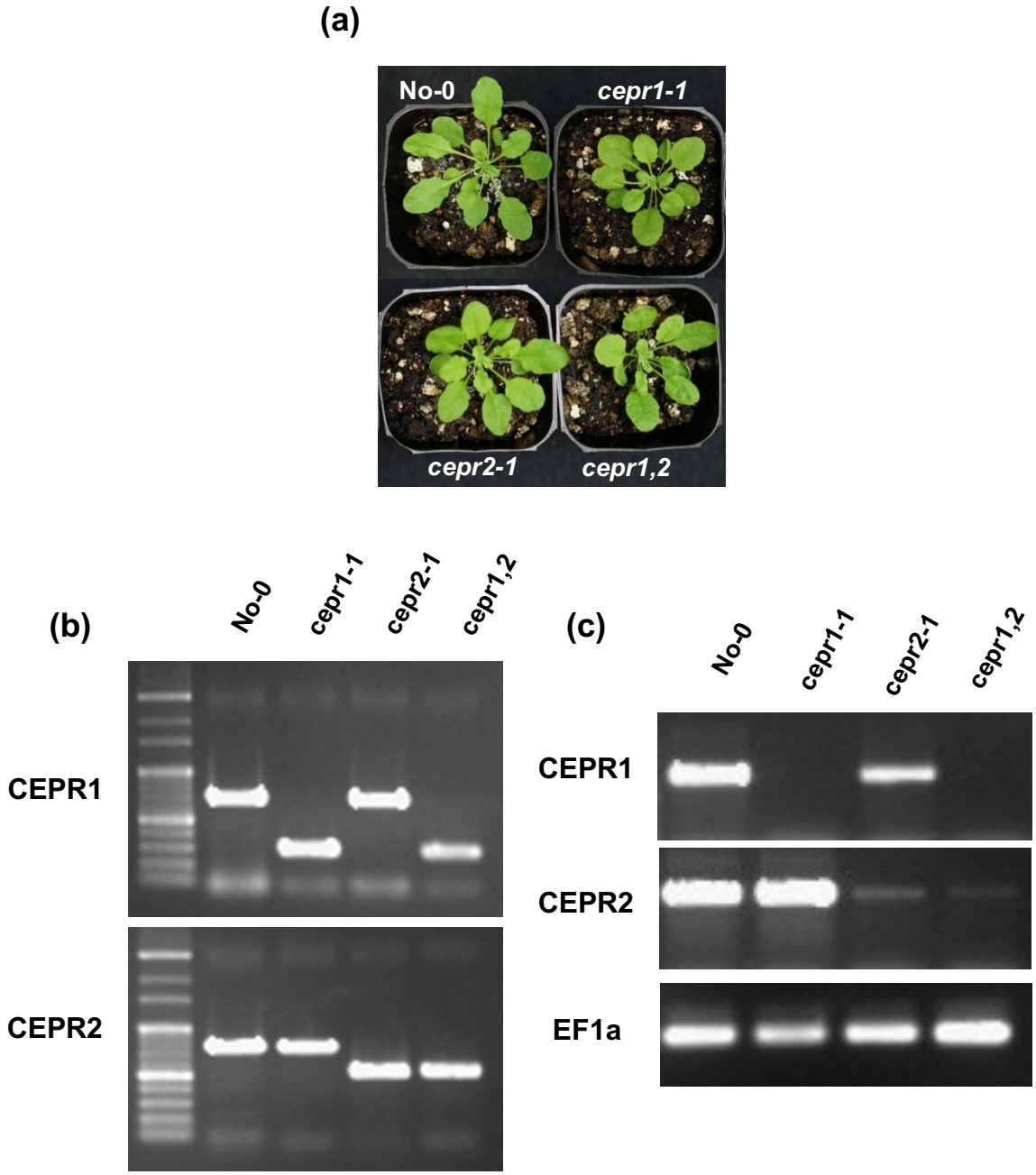



**Fig. S3.4 Identification of *cepr* mutants using RT-PCR**

(a) Four-week-old No-0 and *cepr* mutant plants.

(b) PCR was performed using genomic DNA as a template and using transposon-derived primers listed in Supplementary Table 2. Amplification derived from a normal gene was observed in the wild type, whereas in the mutant of CEPR1 or CEPR2, primers derived from the transposon and one gene specific primer was amplified as a band. In addition, no amplification products from normal genes were observed in *cepr1-1 cepr2-1* mutant.

(c) The expression of CEPR genes confirmed by RT-PCR. Transcripts were detected in wild type, but it was almost completely suppressed in the mutants.

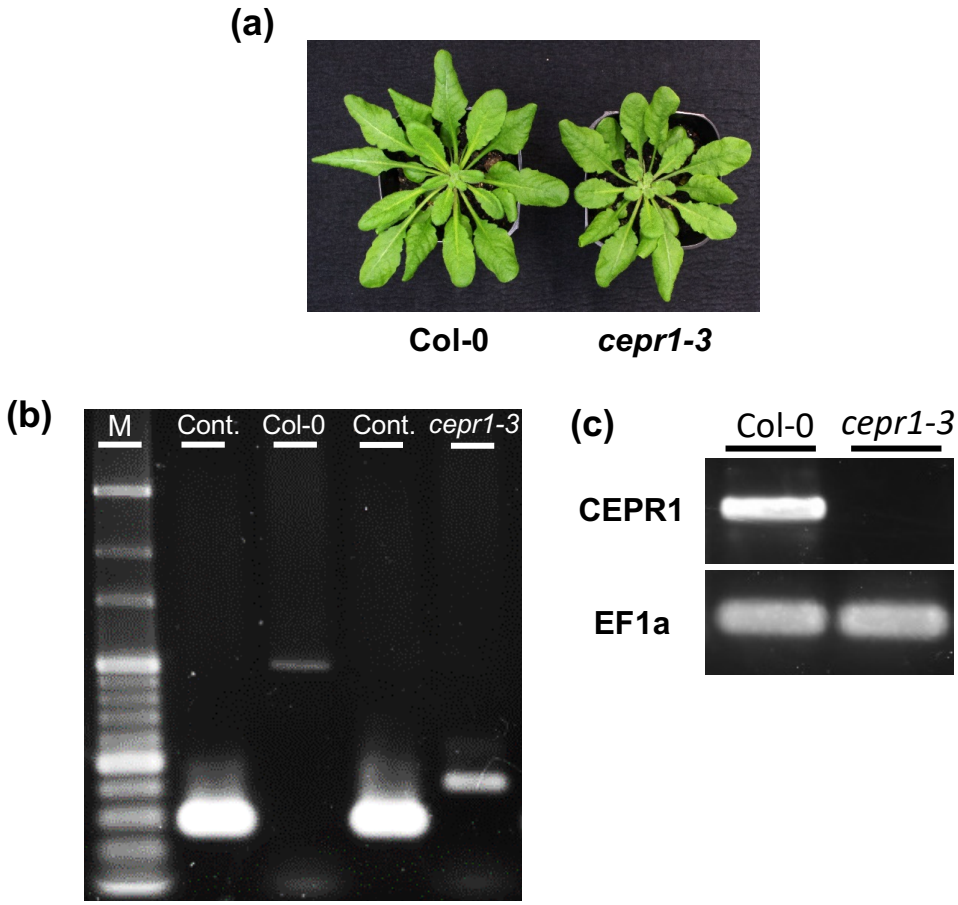



**Fig. S3.5 Identification of *cepr1-3* mutant plant using RT-PCR.**

(a) Five-week-old Col-0 and *cepr1-3* mutant.

(b) PCR was performed using genomic DNA as a template and using transposon-derived primers. Amplification derived from a normal gene was observed in the wild type, whereas in the mutant of CEPR1 primers derived from the transposon and one gene specific primer was amplified as a band. In addition, no amplification products from normal genes were observed in *cepr1-3* mutant.

(c) The expression of CEPR genes confirmed by RT-PCR. Transcripts were detected in Arabidopsis Col-0, but completely suppressed in the *cepr1-3* mutant.

# Chapter 4: Expression of CEP peptide-encoding genes in response to bacterial pathogens


## Abstract

C-terminally encoded peptide (CEP) peptide-encoding genes comprise of *CEP1* to *CEP15*. These genes are known to be perturbed by environmental cues e.g., low N condition. We found that the expression level of certain CEP peptide-encoding genes was upregulated 24 h upon inoculation with avirulent bacteria, *Pseudomonas syringae* pv. *tomato* DC3000 *hrcC*$^{-}$ (*hrcC*$^{-}$) or *Pseudomonas syringae* pv. DC3000 *avrRpm1* (*avrRpm1*) and non-adapted bacterial pathogen, *Pseudomonas syringae* pv. *tabaci* 6605 (*Pta* 6605). Interestingly, 24 h upon inoculation with virulent *Pseudomonas syringae* pv. *tomato* DC3000 (DC3000), *CEP14*, is the only upregulated CEP peptide-encoding gene, raising further question whether CEP14 is also play a role in modulating defense responses.


**Keywords:** c-terminally encoded peptide (CEP), CEP peptide-encoding genes, bacterial pathogen, defense modulation.

## 4.1 Introduction

C-terminally encoded peptides (CEPs) were described as post-translationally modified peptides consisting of 15 amino acids long and usually hydroxylated on the proline residues (Ohyama et al. 2008; Roberts et al. 2013). CEP peptide is well characterized as a root-to-shoot signal peptide that sense nitrogen deficiency and subsequently compensates from N-rich roots ecosystem through systemic signaling (Tabata et al. 2014). Expressed CEP peptides from the root are perceived by typical LRR-receptor like kinases, CEPR1 or CEPR2 (Tabata et al. 2014; Ohkubo et al. 2017). Thus, CEP family have also already been shown to regulate primary and lateral root growth (Delay et al 2013; Roberts et al. 2013).

In Arabidopsis, CEP peptide-encoding genes comprise of a large family (*CEP1* to *CEP15*) and expressed throughout Arabidopsis (Roberts et al. 2013). The expression level of CEP peptide-encoding genes has been previously reported changes under several hormonal and nutritional stimuli e.g., salicylic acid (SA) or under low N condition (Delay et al. 2013; Roberts et al. 2013). However, little is known whether biotic stimuli like bacterial pathogens affect CEP genes expression.

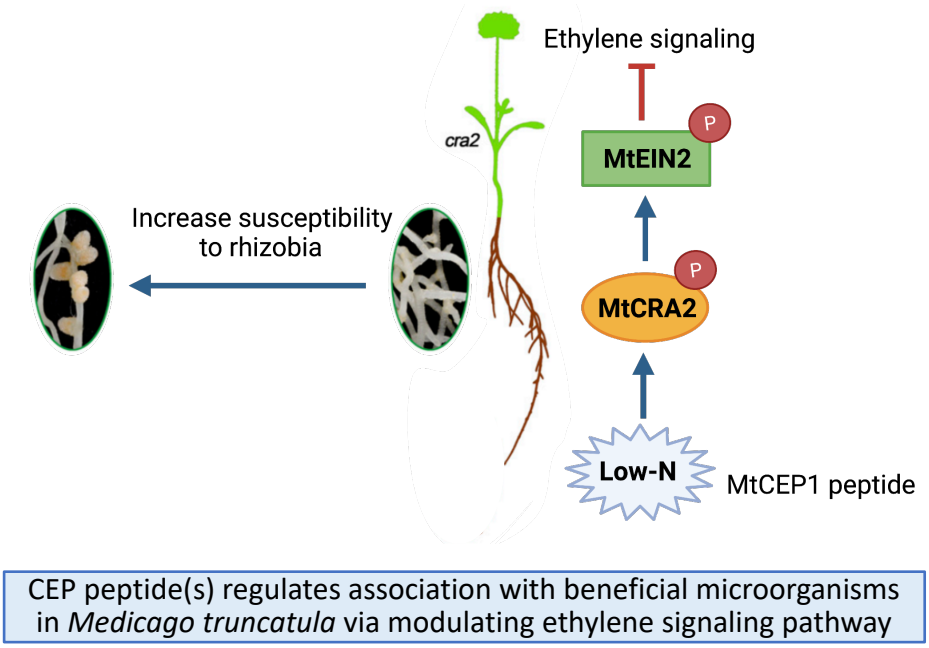



**Fig. 4.1 Model of action of MtCRA2 in regulating root nodulation and symbiotic association under Low-N conditions.** Adapted from Zhu et al. (2020), created with BioRender.com.

Zhu et al. (2020) have shown that in *Medicago truncatula* under low nitrogen condition, a certain CEP peptide activates the MtCRA2 receptor-like kinase, the CEP peptide receptor in *Medicago truncatula*. The MtCEP-activated MtCRA2 can transphosphorylate MtEIN2, the regulator

of ethylene signaling, leading to the repression of ethylene signaling pathway (Zhu et al. 2020). Thus, this repression resulting in increased number of root nodule formation, and also increased susceptibility to rhizobial infection.

In chapter 3, we have shown that exogenous application of CEP5 peptide can enhance disease susceptibility in Arabidopsis against bacterial pathogens and also can suppress Arabidopsis defense responses. Based on that, in this chapter, we try to investigate the role of CEPs in modulating Arabidopsis immunity by analyzing the expression level of CEP peptide-encoding genes upon bacterial pathogens inoculation.

## 4.2 Material and methods

*Arabidopsis thaliana* ecotype Columbia-0 (Col-0) was used in this study and the Col-0 seeds were sown on wetted Jiffy-7 peat pellets (AS Jiffy Products, Oslo, Norway) in the growth chamber for 2 weeks. Germinated seeds were transplanted into soil mixed with vermiculite (1:1) and grown as described previously by Mai et al. (2020). Germinated seeds were transplanted into the mixture of soil and vermiculite (1:1) and grown as described by Mai et al. (2020).

For examination of the expression of CEP peptide-encoding genes, three fully expanded leaves of 5-week-old plant were syringe-infiltrated with virulent bacteria, *Pseudomonas syringae* pv. *tomato* DC3000 (DC3000); avirulent bacteria, *Pseudomonas syringae* pv. *tomato* DC3000 *hrcC*$^-$ (*hrcC*$^-$) or *Pseudomonas syringae* pv. DC3000 *avrRpm1* (*avrRpm1*) and non-adapted bacterial pathogen, *Pseudomonas syringae* pv. *tabaci* 6605 (*Pta* 6605). The inoculum density was adjusted to 2 x $10^6$ cfu/ml for virulent and avirulent bacteria whilst for non-adapted bacteria it was adjusted to 4 x $10^6$ cfu/ml. Water-treated leaves were used as a control treatment. Additional treatment was also carried out by syringe-infiltrated Col-0 leaves with 1 µM flg22 as one of the typical pathogen-associate molecular patterns (PAMPs). The inoculated plants were kept under high humidity for 6 or 24 h before collected for RNA extraction. Total RNA was transcribed as previously described (Mai et al. 2020; Phuong et al. 2020). The qRT-PCR was performed with a Shimadzu GVP-9600 Gene Detection System (Shimadzu, Kyoto, Japan), using a KAPA SYBR FAST Universal qPCR mix (KAPA Biosystems, Boston, MA, USA). Primers were listed in supplementary **Table S4.1** and *EF1-α* (*At5g60390*) was used as a reference gene.

## 4.3 Results and discussions

First, to investigate whether biotic stimuli like bacterial pathogens affect CEP peptide-encoding genes, we analyze the expression level of all CEP peptide-encoding genes upon bacterial pathogens infection. As shown in **Fig. 4.2a**, several CEP genes were significantly upregulated 24 h upon inoculation with non-adapted bacterial pathogen, *Pta* 6605, as indicated by the red box plot graphs compared with water treated leaves. Similar to the expression level of CEP peptide-encoding genes upon *Pta* 6605 inoculation, certain CEP peptide-encoding genes were significantly upregulated compared to water treated leaves, 24 h upon inoculation with avirulent bacteria, *avrRpm1* (**Fig. 4.2b**). Interestingly, 6 h upon inoculation with *avrRpm1*, unlike *Pta* 6605, *CEP14* was also upregulated.

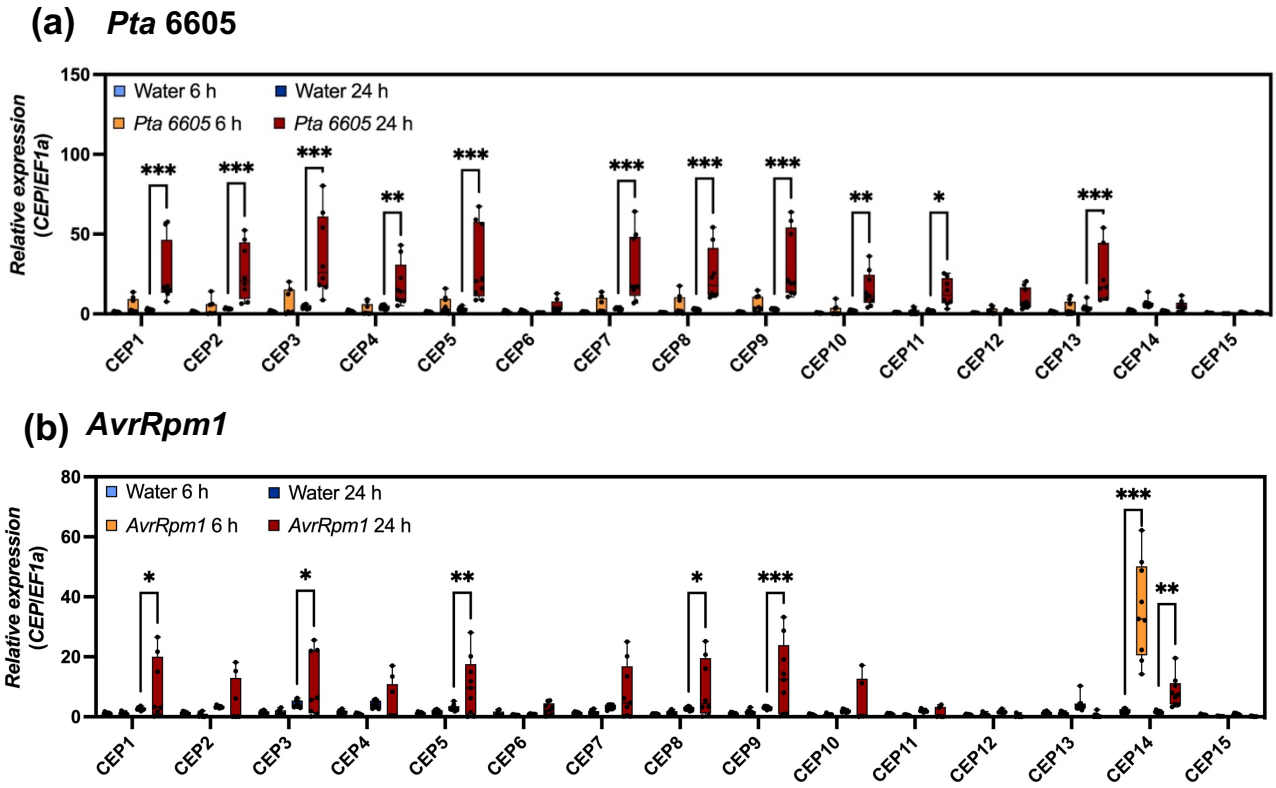



**Fig. 4.2 The expression level of CEP peptide-encoding genes challenged with *avrRpm1* and *Pta 6605*.** Leaves of 5-week-old Col-0 were syringe-infiltrated with a bacterial suspension of *avrRpm1* ($OD_{600}$: 0.001) **(a)** or *Pta* 6605 ($OD_{600}$: 0.002) **(b)** and incubated for 6 and 24 h before subjected for gene expression analysis. Water treated leaves were used as control. Error bars represent means and SDs of three biological replicates. The experiment was repeated two times with similar results; representative results are shown. Asterisks indicate significant difference among treatments using two-way ANOVA with Tukey's multiple comparison test (* $p < 0.033$; ** $p < 0.002$; *** $p < 0.001$).

We then observed the expression level of CEP peptide-encoding genes upon inoculation with another avirulent bacteria, *hrcC*$^-$ and virulent bacteria DC3000. While inoculation with avirulent *hrcC*$^-$ upregulated the expression level of several CEP peptide-encoding genes 24 h post syringe infiltration (**Fig. 4.3a**), almost no upregulation of CEP peptide-encoding genes can be seen upon inoculation by virulent DC3000 (**Fig. 4.3b**). However, only *CEP14* was significantly upregulated 24 h upon inoculation by virulent DC3000 compared with water treated leaves.

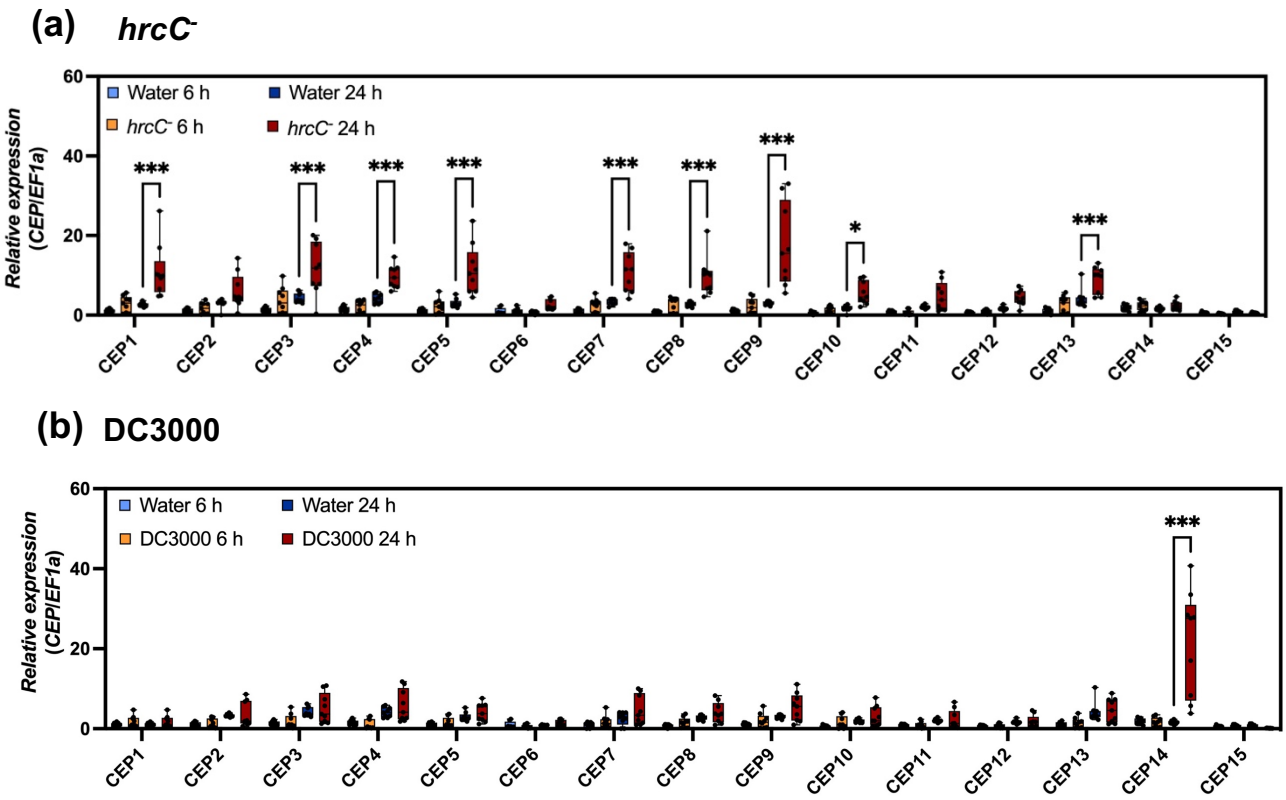



**Fig. 4.2 The expression level of CEP genes challenged with DC3000 and *hrcC*$^-$**. Leaves of 5-week-old Col-0 were syringe-infiltrated with a bacterial suspension of DC3000 or *hrcC*$^-$ ($OD_{600}$: 0.001) and incubated for 6 and 24 h before subjected for gene expression analysis. Water treated leaves were used as control. Error bars represent means and SDs of three biological replicates. The experiment was repeated two times with similar results; representative results are shown. Asterisks indicate significant difference among treatments using two-way ANOVA with Tukey's multiple comparison test (* $p < 0.033$; *** $p < 0.001$).

We next investigated whether CEP genes upregulation also can be seen upon treatment with bacteria flagellin peptide, flg22, one of the typical PAMPs. As we can see in **Fig 4.4**, although the expression level of CEP peptide-encoding genes upon infiltration with flg22 was not as high as bacterial pathogens inoculation, similar upregulation of several CEP genes can be seen 24 h post

syringe infiltration. Furthermore, similar to avirulent *avrRpm1*, we can see the upregulation of *CEP14*, 6 h post syringe infiltration with flg22 compared with water treated leaves.

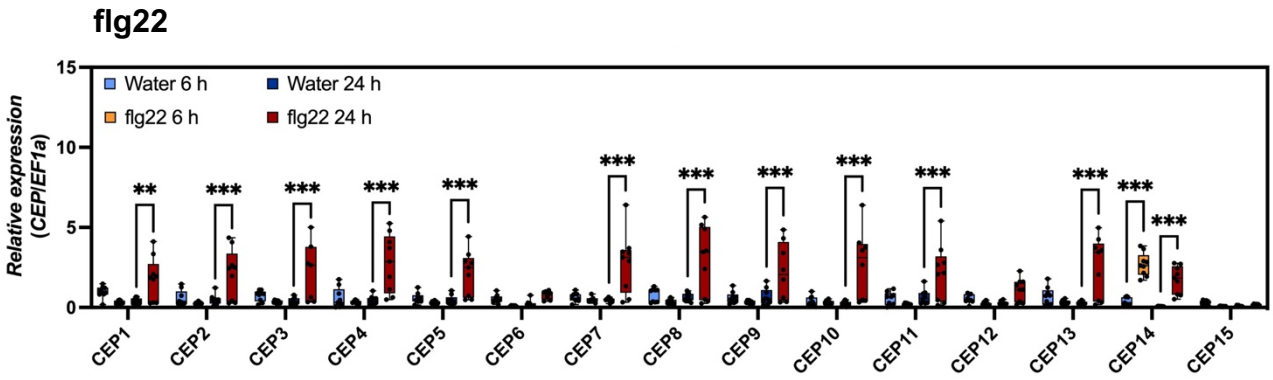



**Fig. 4.4 The expression level of CEP genes challenged with flg22.** Leaves of 5-week-old Col-0 were syringe-infiltrated with flg22 at 1 µM. Water treated leaves were used as control. Error bars represent means and SDs of three biological replicates. The experiment was repeated two times with similar results; representative results are shown. Asterisks indicate significant difference among treatments using two-way ANOVA with Tukey's multiple comparison test (* $p < 0.033$; *** $p < 0.001$).

In this study, we demonstrated that upon bacterial pathogens inoculation and flg22 treatment certain CEP peptide-encoding genes were upregulated (**Fig. 4.2-4.4**). As shown in the heat map visualization (**Fig. 4.5**), *CEP6*, *CEP12* and *CEP15* show no upregulation. Except with the virulent DC3000, *CEP1*, *CEP3*, *CEP5*, *CEP8* and *CEP9* genes were upregulated at 24 h after inoculation, some of which were shown to be induced in response to flg22. Furthermore, 24 h post inoculation with virulent DC3000, *CEP14*, is the only upregulated CEP gene.

As explained in the general introduction (Chapter 1), CEP peptides are divided into two groups based on amino acid similarity: group I consists of CEP1-12 and group II consists of CEP13-15 (Roberts et al. 2013). Here in this chapter, we have shown that certain CEP genes belonging to group I were upregulated as well as from group II upon infection with avirulent or non-adapted bacterial pathogens. Furthermore, as *CEP14* from Group II is upregulated 24 h upon virulent DC3000 inoculation, there is a possibility that CEP14 may play a role in modulating expanded resistance during or in susceptible interaction in Arabidopsis.

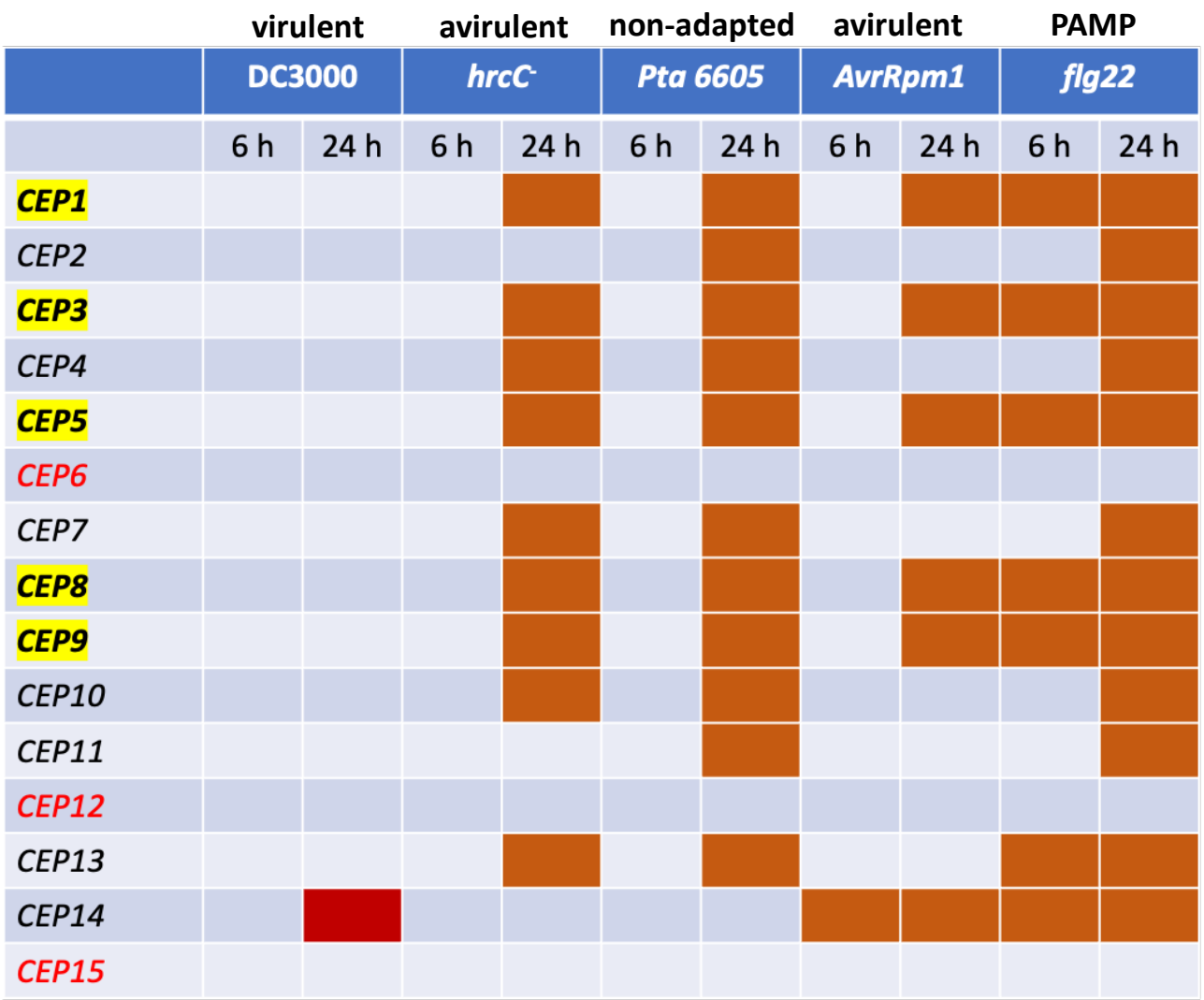



**Fig. 4.5 Heat map and a result of CEP peptide-encoding genes analysis upon bacterial pathogens inoculation and flg22 treatment.** Yellow highlighting indicates the upregulated CEP genes 24 h upon bacterial pathogens inoculation (except virulent DC3000) and flg22 treatment, whilst red-colored font indicates CEP genes with no induction of the expression level upon bacterial pathogens inoculation or flg22 treatment.

## 4.4 Supplementary information

**Table S4.1** Primer sequences used for qRT-PCR

| Gene | Accession | Sequence (5'>3') | References |
|---|---|---|---|
| *CEP1* | *At1g47485* | F: GAGGCCGAGCATCCTAGCAT<br>R: GTCGCCCGTTAGAGTGTCCA | This study |
| *CEP2* | *At1g59835* | F: CCGTGGTGACCATTTTGACC<br>R: CATTCACAACTCCTGGATGC | This study |
| *CEP3* | *At2g23440* | F: GACCTACGGAACCTGGTCAT<br>R: AAAAAGTCACCAGGCCAATC | Delay et al. 2019 |
| *CEP4* | *At2g35612* | F: GTCAAGTGATGGTGTCTCGC<br>R: TTTAAGGAGCACCTGGAGGG | This study |
| *CEP5* | *At5g66815* | F: CCATGGACGAACCCTAAAAG<br>R: TGCCATCATCGTCTTGCTAT | Roberts et al. 2016 |
| *CEP6* | *At5g66816* | F: GTTTACGGAGGCGAGACAGT<br>R: ACCCGACTGTGAAATGATGA | Chapman et al. 2019 |
| *CEP7* | Between *At5g66817* – *At5g66820* | F: GTCGACCTTTGAAGTCATCGC<br>R: AGGGTTGGTAGGCCGAAATG | Chapman et al. 2019 |
| *CEP8* | Between *At5g66817* – *At5g66820* | F: GGACCCATAGAAAGTCAATCAAG<br>R: GCCATGTTCTTCTTTGCTAATGT | Chapman et al. 2019 |
| *CEP9* | *At3g50610* | F: ACGATTGCAGAAAATGAACG<br>R: CTGGAGTTGTAGGTGCGAAA | Chapman et al. 2019 |

| | | | |
|---|---|---|---|
| *CEP10* | Between *At1g36040* – *At1g36050* | F: GGACCGTCATGAGCATTTCA<br>R: CTTAGATGCCCAATACCAGG | This study |
| *CEP11* | Between *At2g23440* – *At2g23450* | F: ATGGCAAAGACACGTCGTGT<br>R: GGTCTTGATCAAGTGGCCAAC | This study |
| *CEP12* | *At1g31670* | F: CCGCCAAGTTCTATCGAGTGG<br>R: GGAGCACCAGGTGGATCCTTG | This study |
| *CEP13* | *At1g16950* | F: GGCTCGTCCAAGGATCTCCA<br>R: CCGGAGGAGAAGTGTGACCC | This study |
| *CEP14* | *At1g29290* | F: GCCGTCATCGTGTCAGCCTT<br>R: GGCCAACACCGGGACTAGGA | This study |
| *CEP15* | *At2g40530* | F: CTCCGGAATTCCTTCGAATC<br>R: ACATGTATGGATCAGTGGCC | This study |
| *EF1α* | *At5g60390* | F: CATCATTTGGCACCCTTCTT<br>R: TGGTGACGCTGGTATGGTTA | Phuong et al. 2020 |

# Chapter 5: General discussions and conclusions

An endogenous suppressor (ES) is defined as a molecule that suppresses elicitor-induced defense responses (Nasu et al. 1992). Recently, we confirmed the presence of ES in *Arabidopsis thaliana* ecotype Col-0, and the partially purified Arabidopsis ES was shown not only to suppress PAMP-induced defense responses, but also to induce susceptibility to non-adapted fungal pathogens (Mai et al. 2020). Based on that, the role of ES is considered to prevent excessive defense responses and fine-tuning the trade-off between growth and defense. Further LC MS/MS analysis of purified Arabidopsis ES identified a peptidic fragment corresponding to c-terminally encoded peptide 5 (CEP5).

CEP peptide(s) is known as a systemic translocation signal for nitrate acquisition that generated from small protein through post-translational modification. CEP peptides usually consist of 15 amino acids with one or more hydroxylated proline (hyp) residue. However, hardly anything is known about CEP peptide activity in plant immunity. Therefore, we synthesized CEP5$^{hyp}$ (DFR{HYP}TT{HYP} GHS{HYP}GIGH; CEP5$^{hyp}$) and examined its activity for defense suppression and its role in plant immunity.

In this study, we showed that Similar to the Arabidopsis ES, CEP5p$^{hyp}$ allowed non-adapted pathogens to cause symptoms, when applied exogenously to Arabidopsis Col-0. *Colletotrichum tropicale* (*Ctro*) or *Mycosphaerella pinodes* (*Mp*), which are pathogenic to mulberry or pea, respectively, did not cause disease symptom on the Col-0 leaves, while symptoms were developed when each conidia were inoculated simultaneously with the CEP5p$^{hyp}$ (**Fig. 2.1**). Notably, on the Col-0 leaves with CEP5p$^{hyp}$, the conidia germinated to form hyphal-like structures, which directly penetrate epidermis without forming melanized appressoria (**Fig. 2.1**). This is reminiscent of the hyphal tip-based entry (HTE) reported by Hiruma *et al*. (2010). *Ctro* employs the HTE on Arabidopsis lack penetration resistance genes (*PEN2* or *PEN3*). Indeed, exogenous CEP5p$^{hyp}$ could suppress the expression of defense related gene associated with preinvasive resistance, *PEN2* but not *PEN3* (**Fig. 2.3**). These findings suggest that exogenous CEP5p$^{hyp}$ targets *PEN2*-, but not *PEN3*-dependent preinvasive resistance in Arabidopsis. In contrast to *PEN2* and *PEN3* expression level, *PDF1.2* was drastically induced in Arabidopsis Col-0 upon inoculation with the mixture of CEP5p$^{hyp}$ and *Ctro* (**Fig. 2.3**). These results indicate that when CEP5 treatment suppressed *PEN2*-dependent preinvasive resistance, postinvasive resistance accompanied by *PDF1.2* expression is immediately induced upon HTE-mediated invasion inside the invaded cells.

Similar induced susceptibility caused by CEP5p$^{hyp}$ was also observed on Arabidopsis Col-0 when challenged with a non-adapted bacterial pathogen *Pta* 6605 (**Fig. 2.4**), a causal agent of the wildfire disease on tobacco. The use of model pathogenic bacteria of DC3000 and its derivatives further revealed that the Col-0 plants with CEP5p$^{hyp}$ also exhibited enhanced disease susceptibility to an avirulent *hrcC*$^{-}$ as well as one expressing *avrRpm1* (**Fig. 3.1**). On the basis of PTI and ETI co-work together, there is possibility that by attenuates PTI, CEP5p$^{hyp}$ indirectly co-suppress ETI responses. Also, there is another possibility that exogenous application of CEP5p$^{hyp}$ also affect ETI responses directly. Taken together, these observations clearly indicate that exogenous CEP5p$^{hyp}$ can suppress the Arabidopsis immunity via affecting the common pathway(s) or process(es) leading to the PTI and ETI.

Further examination revealed that, exogenous CEP5p$^{hyp}$ to Arabidopsis Col-0 caused a suppression or delay both in flg22-induced ROS generation and MAP kinase activation, eventually resulting in insufficient expression of defense-related genes (**Fig. 3.2-3.5**). Indeed, the concomitant presence of flg22 with CEP5p$^{hyp}$ clearly reduced the callose accumulation, a typical marker of PTI (**Fig. 3.6**). It is thus considered again that CEP5p$^{hyp}$ can modulate Arabidopsis immunity by targeting the common defense signaling pathway (**Fig. 5.1**).

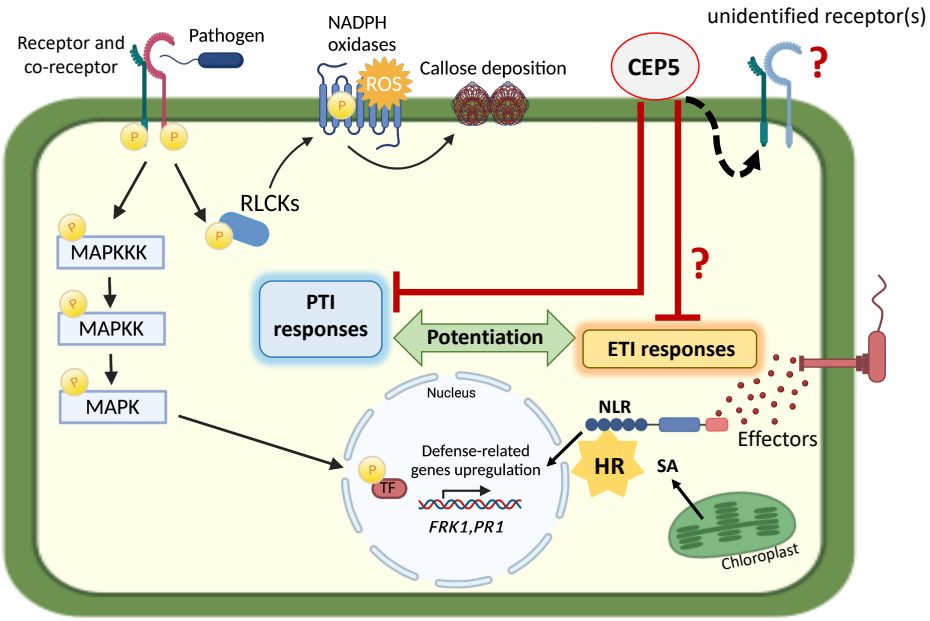



**Fig. 5.1 Model for the action of CEP5p$^{hyp}$ in the suppression of defense responses in the model plant *Arabidopsis thaliana*.** Created with BioRender.com.

CEPs are known to bind to two CEP receptors, CEPR1/XIP1 and CEPR2, during N-demand signaling (Tabata et al. 2014; Ohkubo et al. 2017). Interestingly, the CEP5p$^{hyp}$-mediated susceptibility

was observed on the No-0 and *cepr* mutants when inoculated with *Ctro* or *Mp* (**Fig. 2.2**). An additional *cepr1-3* with a different mutation allele in the Col-0 background also showed the same phenotype. Indeed, CEP5-mediated defense suppression was also observed on *cepr1* and *cepr2* mutants (**Fig. 3.7**; **Fig. S3.3**). Based on these results, it is likely that the signaling pathway underlying the CEP5p$^{hyp}$-caused defense suppression is mediated independently of CEPR1 and CEPR2, which are known to act on systemic signaling during N deficiency. Rather, an unidentified receptor(s) other than CEPR1 and CEPR2 may play a role the CEP5p$^{hyp}$-mediated disease susceptibility (**Fig. 5.1**).

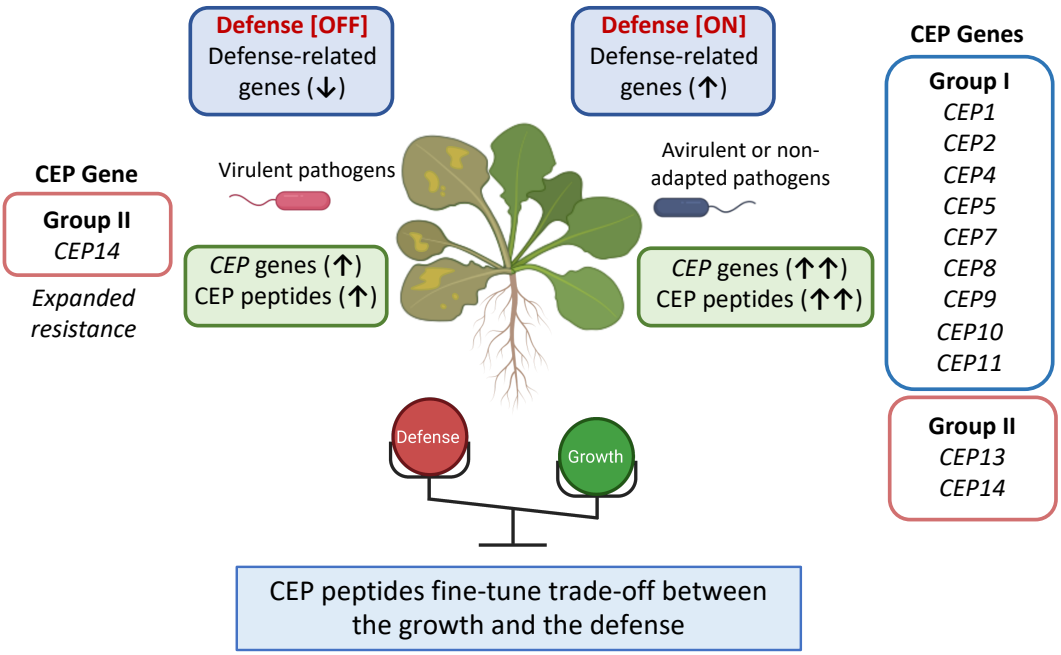



**Fig. 5.2 Model of action of CEP peptide-encoding genes during bacterial pathogens attack.** Created with BioRender.com.

Finally, to understand the role of CEP peptides in modulating the Arabidopsis immunity, expression for *CEP* genes (*CEP1* to *CEP15*) were analyzed with Col-0 leaves challenged with virulent DC3000, avirulent *hrcC*$^{-}$ or *avrRpm1* and non-adapted *Pta* 6605. Upon attack by avirulent or non-adapted pathogens, certain CEP genes belong to group I were upregulated as well as from group II, eventually resulting in the trade-off between growth and defense (**Fig. 5.2**). However, during virulent pathogens invasion, as these virulent pathogens are capable to arrest defense responses most of CEP genes expression are not changing resulting in almost no CEP peptides production and successful infection by virulent pathogens. Furthermore, during susceptible interaction only *CEP14* from Group II upregulated on the later stage of infection, we assume that *CEP14* from Group II also

involved in defense modulation (**Fig. 5.2**). These results support the hypothesis that CEP peptides prevent excessive defenses and fine-tune the trade-off between the growth and the defense. So far, studies on the activation of plant immunity, especially focusing on endogenous SA, have been extensively carried out, but little is known about the endogenous molecule(s) that negatively control plant immunity.

In recent year, in *Medicago truncatula* under high nitrogen condition, a certain CEP peptide, whose is induced in response to symbiotic association or Nod factor-treatment, is shown to regulate locally or systemically secondary infection of rhizobia via affecting ethylene signaling pathway (Zhu et al. 2020). It is thus now becoming clear that CEP peptides regulate not only plant growth, but also their association with microorganisms. In this scenario, individual CEP peptides from a huge gene family (yielding at least 24 peptides in Arabidopsis), likely play diverse roles i.e., growth, symbiotic association, and immunity. This study is thus expected to be a breakthrough in the field of plant-microbe interactions to open a new perspective on the regulatory mechanism of plant immunity.

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