Studies on an effector NLP1 expressed during the late phase of plant infection by *Colletotrichum orbiculare*

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2018

Contents

General introduction

Food security has become the worldwide issue and many countries are experiencing price spikes in the major food staples. The increases in the food prices are partially due to the effect of plant diseases. It has been cited that between 31% to 45% of the total annual worldwide crop are lost to diseases, insects, and weeds together. Around 14.1% of the annual worldwide crops are lost to plant disease alone throughout the world (Agrios, 2004). Examples of plant disease pathogens are including fungi, bacteria, phytoplasmas, viruses and viroids. For the past century, people depended heavily on pesticides to control the plant diseases. However, the extensive use of these toxic chemical brings more harm towards the environment. Therefore, controlling plant disease is one of the important problems for securing healthy and stable food supply and at the same time safeguarding our environment.

Colletotrichum is a genus of major plant pathogens causing anthracnose diseases in many plant crops worldwide. The genus belongs in Ascomycota division, under Sordariomycetes and it comprises more than 100 species that infect a wide range of plant hosts including vegetables, fruits, and cereals (Cannon et al., 2012). It causes dark, sunken necrotic lesion on leaves, stems, flowers and fruits (Agrios, 2004). *Colletotrichum orbiculare* causes anthracnose disease in Cucurbitaceae such as cucumber, and melon. The disease symptoms are including lesions on leaves and fruit rot. Aside of Cucurbitaceae, *C. orbiculare* has been reported to infect *Nicotiana benthamiana* (Shen et al., 2001) which is distantly related to cucumber. One of the feature of *C. orbiculare* during infection process is the formation of specialized infection structure called appressoria that are pigmented with melanin (Kubo and Takano, 2013). It is known that melanin biosynthesis in appressoria is critical for the function of this structure (Kubo and Furusawa, 1991). After the formation of appressoria, *C. orbiculare* exhibits

hemibiotrophic lifestyle which means it have two distinct stages during life cycle (O'Connell et al., 2012; Perfect et al., 1999). Penetration peg emerges from the melanized appressoria and develops primary biotrophic hyphae. This stage is called biotrophic phase i.e., the pathogen will colonize the host and take its nutrients without killing the host cell. Then, the pathogen will switch to the necrotrophic phase by the formation of the secondary invasive hyphae (O'Connell et al., 1985; Latunde-Dada et al., 1996). During this stage, the pathogen starts to kill the host cell, at least partially via production of toxin-like virulence effectors (Kleemann et al., 2012; O'Connell et al., 2012).

Effectors are proteins expressed by plant pathogens to enhance infection of specific plant species. Plants recognize pathogen by sensing Microbe-Associated Molecular Patterns (MAMPs). Once plant recognizes MAMPs, plants mount the defense responses to the pathogen, that is MAMP-triggered immunity (MTI). One important role of effectors is the suppression of MTI, although effectors are likely to have various roles for pathogens. Effectors are often recognized by nucleotide-binding leucine rich repeat (NB-LRR) proteins. This type of recognition event triggers defense responses called as effector- triggered immunity (ETI) (Jones and Dangl, 2006). Filamentous pathogens can possess large collections of effectors and they are differentially expressed during the course of infection or in a histologically specific manner (Toruño et al., 2016). The whole gene expression analysis on *C. orbiculare* using microarray indicate that the expression pattern of effector-like genes is largely changed in the course of fungal infection, suggesting that *C. orbiculare* expresses specific effector sets during distinct pathogenic stages (Gan et al., 2013).

C. orbiculare preferentially expresses a necrosis and ethylene-inducing peptide 1 (Nep1)-like protein named NLP1 during the switch to necrotrophy (Irieda et al., 2014). Nep1-like proteins (NLPs) are widely distributed and they have been identified in many nonpathogenic and pathogenic bacteria, fungi and oomycetes (Gijzen and Nürnberger,

2006; Oome and Van den Ackerveken, 2014). Cytotoxic NLPs are generally expressed at late infection phase of fungi and oomycetes. For example, PsojNip (*Phytophtora sojae* necrosis inducing protein) is expressed exclusively during late stages of the infection, corresponding to the transition to the necrotrophic phase (Dong et al., 2012). It is therefore speculated that cytotoxic NLPs contribute to necrotrophy by killing the host plants. Therefore, I hypothesized that the expression of cytotoxic NLPs must be suppressed at an early infection of each pathogen to establish biotrophy; in other words, the inappropriate expression of cytotoxic NLPs might have negative impacts on the full virulence of the pathogens. Also, several observations have shown that some NLPs are unable to cause necrosis, indicating that these NLPs may have roles independent of cytotoxicity. For example, NLPs without cytotoxicity have been identified in pathogens colonizing monocotyledonous plants (Staats et al., 2007; Motteram et al., 2009; Fang et al., 2017) and obligate biotrophic pathogen (Cabral et al., 2012).

In this thesis, I focused on the NLP effector conserved in broad range of microorganism, especially the stage-specific expression of *C. orbiculare* NLP1. In Chapter I, I investigated the effects of inappropriate expression of NLP1 on *C. orbiculare* virulence. I generated *C. orbiculare* transgenic strains constitutively expressing the cytotoxic protein NLP1 and investigated its effects on pathogen infection. I found that *NLP1* is preferentially expressed in late infection phase of *C. orbiculare* and have cytotoxic activity in *Nicotiana benthamiana.* Constitutive expression of NLP1 in *C. orbiculare* blocks pathogen infection on multiple Cucurbitaceae cultivars via their defense activation. Then, I further studied how secreted NLP1 activates cucumber defense. I discovered that the NLP1-dependent inhibition of pathogen infection is not related with its cytotoxic activity and the MAMP sequence recognized by Arabidopsis. Surprisingly, I found that the C-terminal region of NLP1 is recognized by Cucurbitaceae cultivars and this recognition then strongly activates the cucurbit defense to terminate *C. orbiculare*

infection. In Chapter II, I studied the role and function of *NLP1* in *C. orbiculare* virulence and also studied the *NLP* homologs of *C. orbiculare*, *Cob_08431* and *Cob_05961* which are expressed at the early phase of infection, in contrast to *NLP1*.

Chapter I

Inappropriate expression of an NLP effector in *Colletotrichum orbiculare* impairs infection on Cucurbitaceae cultivars via plant recognition of the C-terminal region

Introduction

Necrosis and ethylene-inducing peptide 1 (Nep1) was originally discovered in the culture filtrate of *Fusarium oxysporum* as a secreted cytotoxic protein that causes cell death concomitant with ethylene production (Bailey, 1995). Nep1-like proteins (NLPs) constitute a superfamily of proteins that are produced by various phytopathogenic prokaryotic and eukaryotic microorganisms (Gijzen and Nürnberger, 2006; Oome and Van den Ackerveken, 2014). Tertiary structural analyses on NLPs of *Pythium aphanidermatum* and *Phytophthora parasitica* revealed structural conservation of the NLPs with cytolytic and pore-forming actinoporins of marine organisms (Ottmann et al., 2009). This data suggests that NLPs destabilize plasma membranes of plants, thereby resulting in plant cell death.

Interestingly, NLPs are also known to stimulate defense responses in dicotyledonous but not in monocotyledonous plants (Boller and Felix, 2009; Gijzen and Nürnberger, 2006; Qutob et al., 2006; Staats et al., 2007). Thus, NLPs are likely to have dual functions in plant-pathogenic microorganism interactions, i.e., triggering immune responses and acting as toxin-like virulence factors. Cytotoxic NLPs activate immunity-related gene expression in *Arabidopsis thaliana*, which clearly overlaps with that induced by flg22 (Bae et al., 2006; Qutob et al., 2006), suggesting the similarity of cytotoxic NLP-triggered defense to MAMP-triggered immunity. Furthermore, whereas the oomycete pathogen *Hyaloperonospora arabidopsidis* produces multiple NLPs, however, all *H. arabidopsidis*

NLPs lack the ability to cause necrosis in dicot plants including the host *A. thaliana* (Cabral et al., 2012) but can induce defense responses such as *PR1* expression in *A. thaliana* (Oome et al., 2014). These suggest at least partial uncoupling of NLP-triggered plant defense activation from their cytotoxicity in *A. thaliana*.

Importantly, a synthetic peptide of 24 aa (called as nlp24) derived from HaNLP3 of *H. arabidopsidis* induces MAMP responses in *A. thaliana*, e.g., ethylene production, and immunity to *H. arabidopsidis* (Oome et al., 2014). In parallel, a synthetic peptide of 20 aa (called as nlp20) derived from PpNLP of *P. parasitica* induces the defense responses of *Arabidopsis* including ethylene production (Böhm et al., 2014). The nlp24 peptide of HaNLP3 is strongly conserved in both cytotoxic and noncytotoxic type 1 NLPs, further confirming that NLPs commonly possess an MAMP signature recognized by *Arabidopsis* (Oome et al., 2014). The sensitivity to nlp20 is detected in some of the *Brassicaceae* species and *Lactuca sativa* (lettuce) (Böhm et al., 2014). Recently, RLP23, the leucine-rich repeat receptor protein, was identified as the receptor of the nlp20 peptide in *A. thaliana* (Albert et al., 2015), revealing that recognition of the nlp MAMP by *A. thaliana* depends on a typical pattern recognition receptor.

Consistent with the finding that *H. arabidopsidis* possesses non-cytotoxic NLPs, 11 out of 19 NLPs derived from an oomycete pathogen *Phytophthora sojae* were shown to lack cytotoxic activities (Dong et al., 2012). *P. sojae* represents a hemibiotrophic lifestyle, i.e., initial biotrophic infection subsequently switching to necrotrophy. Importantly, cytotoxic *NLP* genes are expressed at the onset of necrotrophic growth whereas non-cytotoxic NLP genes are expressed preferentially during biotrophic infection, suggesting functional diversification among the NLPs of *P. sojae* (Qutob et al., 2002; Dong et al., 2012). This tendency is clear in NLPs of an ascomycete fungal pathogen *Colletotrichum higginsianum* which represents a hemibiotrophic lifestyle (Kleemann et al., 2012; O'Connell et al., 2012; Perfect et al., 1999).

The ascomycete *Colletotrichum* species infect a wide range of plant species including many important crops, although each species generally exhibits a narrow and specific host range. *Colletotrichum* fungi develop specialized infection structures called appressoria that are darkly pigmented with melanin (Kubo and Takano, 2013). The genome sequence analyses of *Colletotrichum orbiculare,* which causes cucumber anthracnose disease, uncovered the presence of multiple NLPs in the pathogen (Gan et al., 2013). Among them, a cytotoxic-type NLP named NLP1 is preferentially expressed at late biotrophic and necrotrophic phases (Irieda et al., 2014), consistent with the case of *C. higginsianum* (Kleemann et al., 2012).

The restricted expression of cytotoxic NLPs in the late infection phase in multiple fungal pathogens raises the possibility that the expression of cytotoxic NLPs must be suppressed at early infection stage for successful infection of each pathogen; in other words, the inappropriate expression of cytotoxic NLPs might have negative impacts on the pathogens' full virulence. To assess this possibility, in this study I generated *C. orbiculare* transgenic strains constitutively expressing the cytotoxic protein NLP1. The generated strains were subsequently inoculated on the host plant cucumber to investigate whether the inappropriate expression of cytotoxic NLP1 interferes with *C. orbiculare* host infection. I found that the constitutive expression of NLP1 in *C. orbiculare* completely blocked the pathogen infection on cucumber, supporting my hypothesis that the expression of cytotoxic NLP must be regulated properly for successful infection of the pathogen. However, I found that the NLP1-dependent virulence suppression is uncoupled from its cytotoxic activity. Surprisingly, further studies revealed that the C-terminal region, but not the region containing nlp24, of the NLP1 protein is recognized by Cucurbitaceae plants, suggesting that this recognition results in strong defense activation that can terminate *C. orbiculare* infection.

Results

Constitutive expression of NLP1 in *C. orbiculare* enhanced the cucumber defense and impaired fungal virulence on cucumber

To generate the *C. orbiculare* transgenic strains constitutively expressing CoNLP1 (hereafter called NLP1) under the *TEF* promoter of *Aureobasidium pullulans* (Vanden Wymelenberg et al., 1997), I transformed the *C. orbiculare* wild-type strain 104-T with a plasmid pBATTEFPNLP1 (see details in Materials and Methods). I inoculated the obtained transformants of *C. orbiculare* on host plant cucumber (*C. sativus*) to assess possible effects of the constitutive expression of NLP1 on fungal virulence (Fig. I-1). Remarkably, the inoculation assay revealed that 6 out of 12 transformants did not develop any lesions on cucumber whereas the WT strain caused severe lesions, indicating that the constitutive expression of NLP1 under the control of the *TEF* promoter impaired fungal virulence of *C. orbiculare* on host cucumber (Fig. I-1). The transgenic lines with a failure of infection, named NLP1CE strains, grew normally on nutrient rich media similar to the non-transgenic WT strain (Fig. I-2). Microscopic analysis showed that the NLP1CE strains failed to develop invasive hyphae effectively when compared with the WT strain (Fig. I-3). The invasion ratio of the NLP1CE strain was less than 10% whereas that of the WT strain was approximately 28% (Fig. I-3). I also found that the length of the invasive hyphae produced by appressoria of the NLP1CE strains was shorter that that by appressoria of the WT strain (Fig. I-4).

These findings raised the possibility that the route of entry for the NLP1CE strains was blocked via enhanced activation of pre-invasive defense in cucumber. To assess this, ROS generation was investigated using DAB staining, and it was found that strong DAB signal

was detected around the inoculation sites of the NLP1CE strains in cucumber whereas the signal was undetectable in that of the WT strain (Fig. I-5). This finding indicates that the NLP1CE strains elicited ROS generation in cucumber. The assay for papillary callose deposition also suggested that appressoria of NLP1CE strains strongly induced callose deposition in cucumber when compared with WT (Fig. I-6). These results suggest that the NLP1CE strains of *C. orbiculare* triggered substantial pre-invasive immune responses of cucumber in comparison with the WT strain.

Because NLP1 is a secreted protein, I next asked whether the secretion of NLP1 is critical for NLP1-dependent reduced virulence on cucumber. To assess this point, I generated the *C. orbiculare* strains expressing NLP1 lacking its signal peptide (NLP1ΔSP) under the control of the *TEF* promoter. The inoculation assay of the strains expressing NLP1ΔSP (NLP1ΔSPCE strains) on cucumber revealed that the NLP1ΔSPCE strains retained the WT-level virulence on cucumber (Fig. I-7). This supports the idea that NLP1 needs to be secreted from the pathogen to trigger pre-invasive immune responses of cucumber.

Multiple cultivars are resistant to *C. orbiculare* constitutively expressing NLP1

Next, I asked whether the NLP1 expression by *C. orbiculare* also affects the pathogen virulence to other susceptible plants in addition to cucumber. I first performed the inoculation assay of the NLP1CE strains in three additional Cucurbitaceae cultivars: melon (*Cucumis melo*), winter melon (*Benincasa hispida*), and long melon (*Cucumis. melo* L. var *utilissimus*) (Fig. I-8). As a result, I found that the NLP1CE strains failed to infect all tested Cucurbitaceae cultivars whereas the WT strain developed severe lesions in these cultivars (Fig. I-8). The results indicate that constitutive expression of NLP1 by *C. orbiculare* enhances the resistance of multiple Cucurbitaceae cultivars toward the

pathogen. It is known that *C. orbiculare,* including the strain 104-T used in this study, infects *Nicotiana benthamiana* belonging to the family Solanaceae (Shen et al. 2001; Takano et al. 2006). Thus, I investigated the effects of NLP1 expression on the virulence of *C. orbiculare* toward *N. benthamiana* via the inoculation assay. In contrast to the case of cucumber, the NLP1CE strains clearly developed lesions in *N. benthamiana*, indicating that the NLP1CE strains retained virulence on *N. benthamiana* (Fig. I-9A). However, the size of the lesions developed by NLP1CE strains was relatively smaller than the size of lesions developed by the WT strain (Fig. I-9B). Thus, the constitutive expression of NLP1 in *C. orbiculare* likely enhanced the resistance of *N. benthamiana* to some degree but was not sufficient to block the infection by *C. orbiculare*.

Cytotoxic activity is not related to NLP1-mediated enhanced defense

As mentioned earlier, it is known that multiple NLPs of other fungi have cytotoxic activity toward *N. benthamiana*. I then investigated whether NLP1 of *C. orbiculare* also induces cell death in *N. benthamiana* by *Agrobacterium*-mediated transient expression of NLP1. The transient expression of NLP1 resulted in lesion development in *N. benthamiana*, showing a cytotoxic activity leading to cell death in the plant (Fig. I-10). I next asked whether this activity is related to the negative effect of constitutive NLP1 expression on *C. orbiculare* virulence. Based on Ottmann et al. (2009), I first generated a mutated NLP1 carrying a mutation changing histidine 127 to alanine that is expected to lose its cytotoxic activity; I then confirmed that the transient expression of this mutant NLP1 (named NLP1H127A) did not cause lesion development in *N. benthamiana*, showing that NLP1H127A lost its cytotoxic activity towards the plant (Fig. I-10A). I then generated the *C. orbiculare* strain constitutively expressing NLP1H127A (named NLP1H127ACE) and inoculated the strain on cucumber. The NLP1H127ACE strain

failed to infect cucumber, which is indistinguishable from the phenotype of the NLP1CE strain (Fig. I-10B). This result indicates that the constitutive expression of NLP1H127A, as well as NLP1, impairs fungal virulence of *C. orbiculare* on cucumber and therefore the cytotoxic activity of NLP1 is unlikely to be coupled with the NLP1-dependent reduction of *C. orbiculare* virulence toward cucumber. Compared with the WT strain, the NLP1H127ACE strain also showed slightly reduced virulence on *N. benthamiana*, similar to the NLP1CE strain (Fig. I-11A).

The nlp24 MAMP sequence recognized by *A. thaliana* is not related to the enhanced defense in cucumber.

It has been recently reported that a peptide sequence relatively conserved inside NLP proteins is recognized as a MAMP by *A. thaliana* (Böhm et al., 2014; Oome et al., 2014). Based on the finding that the expression of NLP1H127A lacking the cytotoxic activity can reduce the virulence of *C. orbiculare* the same as the expression of the intact NLP1, I next investigated the relationship of the MAMP sequence of NLP1 to the negative impact on virulence. It has been reported that the sequence AIMYAWYFPKDSPMLLMGHRHDWE (Hanlp24) in HaNLP3 of *H. arabidopsidis* is recognized by *A. thaliana* and solely elicits the defense responses of *A. thaliana* (Fig. I-12A; Oome et al., 2014). It was also reported that the corresponding region in *Pp*NLP of *P. parasitica* (AIMYSWYFPKDSPVTGLGHRHDWE) is recognized by *A. thaliana* accompanied with activation of the defense responses (Böhm et al., 2014). *C. orbiculare* NLP1 also has a typical MAMP sequence (AIMYSWYMPKDSPSTGLGHRHEWE, named Conlp24) and I found that the synthetic peptide for Conlp24 indeed elicits ROS generation in *A. thaliana* (Fig. I-12B), similar to the previous report on ROS generation by the nlp20 peptides of *Pp*NLP in *A. thaliana* (Böhm et al., 2014; Albert et al., 2015). I

then generated a mutant NLP1 lacking the nlp24 MAMP sequence. Based on the reported mutational analyses to nlp20 of *Pp*NLP (Böhm et al., 2014), I decided to substitute isoleucine (position 2), methionine (position 3), and tyrosine (position 4) in Conlp24 with alanine, designated as Conlp24Mut, and confirmed that the synthetic peptide for Conlp24Mut failed to elicit ROS generation in *A. thaliana* (Fig. I-12B). I then generated *C. orbiculare* strains expressing the mutated NLP1 having the corresponding mutations (named NLP1Mut) and the generated *C. orbiculare* strains designated NLP1MutCE were subjected to inoculation assay on cucumber. The *C. orbiculare* NLP1MutCE strains were still not able to infect cucumber as with the NLP1CE strains (Fig. I-12C). Collectively, the findings suggest that the negative effect of NLP1 expression on *C. orbiculare* virulence is uncoupled from the reported nlp20/24 MAMP sequence and its cytotoxic activity in NLP1. The NLP1MutCE strain also had slightly reduced virulence on *N. benthamiana* the same as the NLP1CE strain (Fig. I-11B).

The carboxyl-terminal region of NLP1 is recognized by Cucurbitaceae cultivars

The findings raised the possibility that secreted NLP1 is recognized by cucumber via a region distinct from Conlp24 sensed by *A. thaliana*. To determine the responsible region, I decided to generate a deletion series of NLP1 that are sequentially deleted from its carboxy terminus, i.e., the deletion of 22 amino acids (aa), 42 aa and 62 aa (Note: all deleted NLP1 forms still retain the reported nlp24 MAMP sequence and the domain for cytotoxic activity) (Fig. I-13A).

I then generated *C. orbiculare* transgenic lines expressing these truncated forms of NLP1 under the control of the *TEF* promoter (Fig. I-13B). I found that the expression of all NLP1Δ22, NLP1Δ42, and NLP1Δ62 proteins had no effects on the virulence of *C. orbiculare*, whereas the expression of full-length NLP1 completely blocked the infection

of *C. orbiculare* on cucumber (Fig. I-13B). The result clearly indicates that the deletion of the 22 aa carboxyl terminal region abolishes the NLP1 effects on *C. orbiculare* virulence to cucumber, and also raises the possibility that the corresponding carboxyl-terminal region of NLP is recognized by cucumber, which leads to the activation of the cucumber defense against the pathogen.

To assess this possibility, I investigated whether the expression of the carboxyl-terminal region in *C. orbiculare* is sufficient for cucumber defense activation. I generated *C. orbiculare* lines that secrete a red fluorescent protein mCherry fused to the C-terminus 32 aa of NLP1 named mCNLP1C32 (Fig. I-14A) because I considered that the addition of mCherry can mimic the possible recognition of the C-terminal region of NLP1 compared with the sole expression of the C-terminal region as a short peptide. I first constructed a fungal transformation vector carrying the signal peptide derived from the effector DN3 (SP):mCherry:NLP1ΔSP, called mCNLP1FL, as a control, and introduced it into *C. orbiculare* (Fig. I-14A). The obtained mCNLP1FL transformants were subjected to inoculation assay on cucumber. Out of 16 tested mCNLP1FL transformants (SP:mCherry:NLP1ΔSP), four transformants clearly exhibited reduced virulence on cucumber (Fig. I-14B). This suggests that the secreted mCherry:NLP1ΔSP activates cucumber defense via its recognition similar to NLP1 without mCherry. I then constructed a fungal transformation vector carrying SP:mCherry:NLP1C32 (Fig. I-14A) and introduced it into *C. orbiculare*. Out of 48 mCNLP1C32 transformants, three transformants failed to infect cucumbers in contrast to the WT strain (Fig. I-14B), which is similar to *C. orbiculare* strains expressing NLP1 or SP:mCherry:NLP1ΔSP. Thus, the finding supports the idea that the 32 aa carboxyl-terminal region of NLP1 (NLP1C32) is recognized by cucumber.

I further assessed why the other 45 mCNLP1C32 transformants of the 48 tested retained virulence on cucumber. I hypothesized that the transformants with full virulence

might exhibit lower expression of NLP1C32 compared with the transformants lacking virulence. Indeed, qRT-PCR analysis of the SP:mCherry:NLP1C32 gene during the pre-invasive infection phase of *C. orbiculare* showed that the expression levels in the three transformants lacking virulence (mCNLP1C32-1 to C32-3) in this infection phase were several-fold higher than that in the transformants with full virulence (mCNLP1C32-4 and C32-5) (Fig. I-14C). The finding strongly suggests that (i) NLP1C32 is recognized by cucumber and (ii) the threshold of the expression level of NLP1C32 is likely to be present to elicit the cucumber defense.

Next, melon and *N. benthamiana* were inoculated with the mCNLP1C32 strains that failed to infect cucumber (Fig. I-15). Importantly, the mCNLP1C32 strains completely lacked virulence on melon but retained virulence on *N. benthamiana* with a slight reduction (Fig. I-15), which was identical to the mCNLP1FL strains and NLP1CE strains that secrete the full-length NLP1. In contrast, I found that a mutant defective in the *CST1* (*Colletotrichum* Ste12 like) gene lacked virulence on both melon and *N. benthamiana* (Fig. I-15), because the *CST1* gene is required for a fundamental process of *C. orbiculare* plant infection (Tsuji et al. 2003). Therefore, the NLP1C32 expression in *C. orbiculare* mimics the unique virulence-related phenotype of *C. orbiculare* constitutively expressing NLP1. Collectively, these findings indicate that the C-terminal region of NLP1 is recognized by Cucurbitaceae cultivars and then strongly activates their defense to terminate *C. orbiculare* infection. The NLP1C32 sequence is highly conserved in NLP1 homologues of other fungi and oomycete (Fig. I-16), implying possible plant recognition of these NLPs at their C-terminal region.

Discussion

In this chapter, I have revealed that the constitutive expression of NLP1 in *C. orbiculare* strongly reduced the virulence of the pathogen towards its host plant cucumber by inducing cucumber defense. Cytotoxic NLPs are generally expressed at the late infection phase such as during the switching phase from biotrophy to necrotrophy (Qutob et al., 2002; Dong et al., 2012). Thus, these findings suggest that the tight regulation of NLP1 expression is important and the inappropriate expression of NLP1 has a negative impact on virulence. In contrast with my results, it was reported that overexpression of a *Fusarium* NLP1 (NEP1) in *Colletotrichum coccodes* resulted in enhanced virulence on *Abutilon theophrasti* and expanded it host range to tomato and tobacco (Amsellem et al., 2002). Importantly, I found that NLP1-mediated reduced virulence does not depend on its cytotoxic activity via characterization of *C. orbiculare* strains expressing NLP1 lacking the cytotoxic activity (Fig. I-10).

I then asked whether the NLP1-mediated reduced virulence is related to the putative nlp24/nlp20 that can be recognized by *A. thaliana* (Böhm et al., 2014; Oome et al., 2014). I first found that the synthetic peptide corresponding to the nlp24 region of *C. orbiculare* NLP1 can activate the defense response of *A. thaliana* (Fig. I-12), confirming that NLP1 contains an active nlp24 MAMP signature. I then generated *C. orbiculare* expressing a mutant NLP1 lacking functional nlp24; analyses on these strains indicated that the reduced virulence mediated by NLP1 was uncoupled from the nlp24 of NLP1 (Fig. I-12). It has been reported that the nlp20 of *Pp*NLP in *P. parasitica* is recognized as a MAMP sequence by some *Brassicaceae* species and *Lactuca sativa* but not by other plants such as *N. benthamiana*, *Triticum aestivum* and *Solanum lycopersicum* (Böhm et al., 2014). My data suggests that the nlp24/nlp20 sequence is also unlikely to be recognized by cucumber.

Through the deletion analyses of NLP1 combined with the mCherry fusion assay, I found that the carboxy-terminal 32 aa of NLP1 is able to elicit cucumber defense via its recognition (Fig. I-14). Importantly, this recognition system is conserved in multiple other Cucurbitaceae plants (Fig. I-8). Expression of NLP1 in *C. orbiculare* also resulted in a slight reduction of virulence on *N. benthamiana* (Fig. I-9 and Fig. I-15). The inoculation assay of *C. orbiculare* expressing the mutated NLPs (H127A and Conlp24Mut) suggested that the reduced virulence mediated by NLP1 on *N. benthamiana* is uncoupled from its cytotoxic activity and the nlp24 sequence (Fig. I-11), i.e., *N. benthamiana* is likely to recognize the carboxyl terminal region of NLP1, however, the plants fail to mount an effective defense against *C. orbiculare* for an unknown reason.

The identified 32 aa sequence of NLP1 (NLP1C32) is relatively conserved in other plant pathogenic fungi and oomycete (Fig. I-16), suggesting the possible recognition of the C-terminal region of the NLPs by plants. It remains to be elucidated whether the application of the synthetic 32 aa peptide of NLP1 is sufficient to activate the defense of Cucurbitaceae plants or not. I cannot exclude the possibility that the tertiary structure of the 32 aa region, including its modification, is necessary for the defense activation. For a detailed understanding of how Cucurbitaceae plants recognize the C-terminal region of NLP1 and mount a strong defense as its output, further studies are essential, including the identification of a corresponding NLP1 receptor in Cucurbitaceae plants.

Collectively, my data indicate different evolutionary processes for NLP recognition among higher plants, for example between *Brassicaceae* and *Cucurbitaceae*. The MAMP sequence of bacterial flagellin (flg22) is known to be recognized by a wide range of plants, but, importantly, it was reported that a different region of bacterial flagellin is also recognized by a part of plants including tomato (Cai et al., 2011; Hind et al., 2016). Thus, my study provides the example that a conserved molecule of fungal and oomycete

pathogens is also recognized at distinct positions by higher plants probably via independent evolution.

The finding also suggests that the strong activation of MAMP-triggered immunity (MTI) is sufficient to block host infection by *C. orbiculare*, especially at the appressorium-mediated host invasion step. Consistently, in non-host interactions, appressoria of *Colletotrichum* species fail to develop invasion hyphae, for example, appressoria of *C. orbiculare* never develop invasive hyphae in non-host *A. thaliana* (Shimada et al., 2006). This type of phenomenon is also observed in the interaction of susceptible plants with *C. orbiculare ssd1* mutants that can over-activate MTI (Tanaka et al., 2007, 2009). Thus, the strong activation of MTI is sufficient to terminate the penetration function of melanized appressoria of *Colletotrichum* species that can generate strong turgor pressure (Bechinger et al., 1999).

However, it is also plausible that the WT strain of *C. orbiculare* suppresses the defense of host cucumber by deploying appropriate effectors whereas the pathogen fails to infect *A. thaliana* because it has no appropriate effectors. The question is why the *C. orbiculare* transgenic lines expressing NLP1 failed to infect cucumber even in the presence of an effective effector set. I assume that the putative effector set might not be sufficient to suppress the strong immune responses triggered by the recognition of NLP1 when constitutively expressed. Consistent with this, it has been reported that the over-expression of a glycoside hydrolase family 12 protein XEG1, recognized as MAMP, inhibited *P. sojae* infection on soybean (Ma et al., 2015). Based on the genome sequence of *C. orbiculare*, the pathogen has seven NLP genes (Gan et al., 2013). Microarray analysis suggested that multiple NLP genes are likely expressed during the early phases of infection (Gan et al., 2013). I found that the corresponding regions for NLP1C32 in the other NLPs of *C. orbiculare* likely exhibit conservation with CoNLP1 to some degree (Fig. I-17), but they also represent increased variations in comparison with the CoNLP1

homologues of other fungi and oomycetes (Fig. I-16). The reason why the expression of these NLP genes during the early infection phase does not elicit strong defense on cucumber might be (i) the expression level is not enough for the elicitation or (ii) their sequence/structure is not recognized by the plant in contrast to NLP1.

Materials and methods

Fungal strains, media, transformation and DNA analysis

Colletotrichum orbiculare (syn. *C. lagenarium*) wild-type (WT) strain 104-T (MAFF240422) is stored at the Laboratory of Plant Pathology, Kyoto University. All *C. orbiculare* strains were maintained on 3.9% (w/v) potato dextrose agar (PDA; Nissui, Tokyo, Japan) at 24ºC in the dark. The transformation of *C. orbiculare* was based on the method in Kimura et al. (2001). Bialaphos-resistant transformants were selected on PDA plates with 25 μg/mL bialaphos (Wako Pure Chemicals, Japan). The total DNA of *C. orbiculare* was isolated from mycelia with the DNeasy plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Plasmid construction

All plasmids used for constitutive expression of NLP1 and its derivatives were constructed using the plasmid pBATTEFP (Asakura et al., 2009). pBATTEFP was generated by introduction of the *TEF* promoter into pBAT (Kimura et al., 2001), a derivative of pCB1531 (Sweigard et al., 1997) that carries the bialaphos resistance gene (*bar*). All primers used in this study are listed in Table I-1.

To constitutively express NLP1 in *C. orbiculare* under the control of the *TEF* promoter, *NLP1* cDNA fused with an HA epitope tag sequence was amplified with primers

TEFpNLP1_f and TEFpNLP1_r. The amplified fragment was digested with *Xba*I and *Eco*RI and introduced into pBATTEFP to produce pBATTEFPNLP1.

To generate mutant NLP1 lacking its cytotoxic activity, one single site-directed mutagenesis was carried out to substitute His127 in *NLP1* with Ala (CAC to GCA) by recombinant PCR using the primer pairs TEFpNLP1_f/H127A_r and H127A_f/TEFpNLP1_r. Then, the full-length fragment of *NLP1*H127A fused with an HA epitope tag was amplified using TEFpNLP1_f and TEFpNLP1_r primers. The amplified fragment of the HA-tagged *NLP1*H127A was digested with *Xba*I and *Eco*RI and then inserted into pBATTEFP, resulting in pBATTEFPNLP1H127A.

For the construction of mutant NLP1 lacking the functional nlp24 sequence, site-directed mutagenesis by primer extension was performed to substitute Ile111, Met112, and Tyr113 with Ala (ATC ATG TAC to GCC GCG GCC). In the first round of PCR, the primer sets NLP1_XbaI_F/NLP1_MAMP_R and NLP1_MAMP_F/TEFpNLP1_r were used to amplify two products with the mutated sequence at a known MAMP region. In the next round of PCR, NLP1_XbaI_F and TEFpNLP1_r primers were used to amplify the full-length of *NLP1*Mut fused with the HA epitope tag which contained the desired mutation. Next, the amplified fragments of *NLP1*Mut fused with the HA epitope tag were digested with *Xba*I and *Eco*RI and then inserted into pBATTEFP, resulting in pBATTEFPNLP1Mut.

To express HA-tagged *NLP1* under the control of the 35S promoter, a *Bam*HI-*Eco*RI fragment of *NLP1* was amplified by primers 35SpNLP1_f and 35SpNLP1_r. The amplified fragment was digested with *Bam*HI and *Eco*RI and then introduced into the *Bam*HI–*Eco*RI site of pBICP35, resulting in pBICP35NLP1. To express HA-tagged *NLP1*H127A under the control of the 35S promoter, site-directed mutagenesis by primer extension was used to substitute His127 with Ala (CAC to GCA) by using the primer pairs 35SpNLP1_f/H127A_r and H127A_f/35SpNLP1_r. Next, 35SpNLP1_f and

35SpNLP1_r primers were used to amplify the full length of *NLP1*H127A. The amplified fragment was digested with *Bam*HI and *Eco*RI and then inserted into the *Bam*HI–*Eco*RI site of pBICP35 (Mori et al., 1991), resulting in pBICP35NLP1H127A.

To clarify the importance of the *NLP1* signal peptide, a plasmid carrying *NLP1* lacking the region encoding the signal peptide (NLP1ΔSP-HA) was constructed. The *NLP1* cDNA lacking the signal peptide was amplified with primers TEFpNLPdSP_f and TEFpNLP1_r. The amplified fragment was digested with *Xba*I and *Eco*RI and introduced into pBATTEFP to produce pBATTEFPNLP1ΔSP.

To identify the NLP1 region that is recognized by cucumber, cDNA fragments of *NLP1*Δ22, *NLP1*Δ42, and *NLP1*Δ62 were amplified by PCR with the following primers: NLP1_XbaI_F/NLP1_del_217_R for *NLP1*Δ22, NLP1_XbaI_F/ NLP1_del_197_R for *NLP1*Δ42, and NLP1_XbaI_F/ NLP1_del_177_R for *NLP1*Δ62. The amplified fragments were digested with *Xba*I and *Eco*RI and introduced into pBATTEFP, resulting in pBATTEFPNLP1Δ22, pBATTEFPNLP1Δ42, and pBATTEFPNLP1Δ62, respectively.

To investigate whether the carboxy terminal 32 aa region of NLP1 is sufficient to trigger cucumber defense, two expression vectors, pBATTEFPmCNLP1FL and pBATTEFPmCNLP1C32, were constructed by using a multi-fragment cloning method. SP^{CoDN3A}mCherry was amplified using pBATTEFPSP^{CoDN3A}-mCherry (Irieda et al. 2014) as a template with the first primer set NLP1_Sp_mCherry_F1/ NLP1_Sp_mCherry_R1. In parallel, the second primer sets, NLP1_217_mCherry_F2/ NLP1_Sp_mCherry_R2 and NLP1_32_mCherry_F2/ NLP1_Sp_mCherry_R2, were designed and used to amplify NLP1ΔSP and 32 amino acids of the carboxy-terminal region of NLP1 fragments, respectively. Each amplified fragment was assembled and inserted into pBATTEFP linearized with *Xba*I and *Eco*RI using the In-Fusion HD Cloning Kit (Takara, Otsu, Japan) according to the manufacturer's protocol.

Inoculation assay of *C. orbiculare*

In the inoculation assay of *C. orbiculare* on cucumber, 10 μL conidial suspensions (5 x 10^5 conidia/mL) were drop-inoculated onto detached cucumber cotyledons (*Cucumis sativus*), melon (*Cucumis melo*), winter melon (*Benincasa hispida*) or long melon (*Cucumis melo* L. var *utilissimus*). Inoculated cotyledons were incubated at 24ºC for 7 days. The cotyledons were picked from 10 day-old plants (cucumber, melon, and long melon) or from 2-week-old plants (winter melon). For inoculation on *N. benthamiana,* true leaves detached from approximately 5-week-old plants were used. Each virulence test was performed at least three times under the same conditions.

Light microscopy

To investigate appressorium-mediated host invasion by *C. orbiculare* or detect reactive oxygen species (ROS) accumulation, 10 μL conidial suspensions (5 x 10^5 conidia/mL distilled water) were spotted onto the abaxial surface of cucumber cotyledons and incubated in a humid chamber at 24ºC. For appressorium-mediated host invasion, I peeled off the lower epidermis of the cotyledons at 4 days post inoculation (dpi) and placed them on glass slides for observation. The invasive hyphae were observed in bright field by using an Olympus BX53 fluorescence microscope (Olympus) equipped with an Olympus DP72 camera and Olympus cellSens software (Lin et al., 2012).

For the detection of ROS accumulation, the abaxial surface of cucumber cotyledons at 2 dpi was soaked in 1 mg/mL 3,3-diaminobenzidine (DAB; Santa Cruz Biotechnology, Dallas, Texas) solution for 24 h at 24ºC in the dark (Fukada et al., 2016). Then, the abaxial surface of stained cotyledons was peeled off and observed with a bright field Olympus BX53 fluorescence microscope (Olympus) equipped with an Olympus DP72 camera and Olympus cellSens software.

A. tumefaciens-mediated transient expression in *N. benthamiana*

N. benthamiana plants (5 weeks old) were used for the agro-infiltration assay. Plants were grown in a controlled environment chamber at 25ºC with 16 h of illumination per day. Each construct (pBICP35NLP1, pBICP35NLP1H127A) was transformed into *Agrobacterium* strain GV3101 pMP90 by electroporation. Each transformant was cultured in YEP containing kanamycin (100 μg/mL), rifampicin (100 μg/mL) and gentamycin (50 μg/mL). The cells were harvested by centrifugation and were suspended in MMA induction buffer (1 L of MMA, 5 g of Murashige-Skoog salts, 1.95 g of MES, 20 g of sucrose and 200 μM acetosyringone, pH 5.6) (Yoshino et al., 2012). All suspensions were incubated for 1–3 h at room temperature prior to infiltration. The suspensions were infiltrated into *N. benthamiana* leaves using a syringe. The infiltrated areas were observed at 5 days after the infiltration.

Detection of oxidative burst in leaf discs of *A. thaliana*

ROS released by leaf tissue was assayed as described (Ranf et al., 2011) using 3 mm leaf discs in 96-well plates containing 0.1 mL distilled water supplied with 20 μM luminal and 1 μg horseradish peroxidase (Sigma-Aldrich) measured in 2 min intervals for 40 min using Luminoskan Ascent 2.1. (Thermo Scientific, Yokohama, Japan).

Quantitative RT-PCR analysis

Cucumber cotyledons were drop-inoculated with 10 μL conidial suspensions (5 x 10^5 conidia/mL) and incubated for 1 day in a humid chamber at 24ºC. Total RNA was extracted using the RNeasy Plant Mini-Kit (Qiagen Hilden, Germany) according to the manufacture's protocol and treated with Promega RQ1 RNase-free DNase (Promega, Madison, Wisconsin) to remove DNA contamination. The first strand cDNA was synthesized using Takara PrimeScript™ RT reagent Kit (Takara, Otsu, Japan), followed

by quantitative PCR with specific primers to nucleotide sequences corresponding to 32 amino acids of the NLP1 C-terminal region as shown in the Supplementary Table S1. The expression of the actin gene of *C. orbiculare* was used as an internal standard. The quantitative PCR was carried out using SYBR premix ExTaq II (Takara, Otsu, Japan) with a Thermal Cycler Dice Real Time system TP800 (Takara).

The assay for the elongation level of primary invasive hyphae

Ten μL conidial suspensions (5 x 10^5 conidia/mL distilled water) were spotted onto the abaxial surface of cucumber cotyledons and incubated in a humid chamber at 24ºC. I peeled off the lower epidermis of the cotyledons at 4 dpi and placed them on glass slides for observation. The invasive hyphae were observed in bright field by using an Olympus BX53 fluorescence microscope (Olympus) equipped with an Olympus DP72 camera and Olympus cellSens software and their elongation level was classified into the three categories.

Callose deposition assay

Ten μL conidial suspensions (5 x 10^5 conidia/mL distilled water) were spotted onto the abaxial surface of cucumber cotyledons and incubated in a humid chamber at 24ºC for 2 days. Then, the lower epidermises of inoculated cucumber cotyledons were peeled off and stained with 0.01% (w/v) aniline blue in 0.15 M K_2HPO_4 (Fukada et al., 2016). Stained samples were observed with an Olympus BX53 fluorescence microscope (Olympus) equipped with an Olympus

Table I-1. Primers used in study in Chapter I.

Name	Sequence 5' to 3'
NLP1 overexpression	
TEFpNLP1_f	GCTCTAGACAGACACAATGGCCCCTTCGCTCTTCC
TEFpNLP1_r	CCGGAATTCTTAAGCGTAATCTGGAACATCGTATGGGTACAAGGCCGCCTTTCCGAGGTTGTC
Site-direct mutagenesis for cytotoxic activity	
TEFpNLP1_f	GCTCTAGACAGACACAATGGCCCCTTCGCTCTTCC
TEFpNLP1_r	CCGGAATTCTTAAGCGTAATCTGGAACATCGTATGGGTACAAGGCCGCCTTTCCGAGGTTGTC
H127A_f	GACGGGCCTCGGCGCACGCCACGAGTGGGA
H127A_r	TCCCACTCGTGGCGTGCGCCGAGGCCCGTC
Expression of NLP1 lacking its signal peptide	
TEFpNLPdSP_f	GCTCTAGACAGACACAATGGCGCCCATCCAGCCCCGTGCCGTC
TEFpNLP1_r	CCGGAATTCTTAAGCGTAATCTGGAACATCGTATGGGTACAAGGCCGCCTTTCCGAGGTTGTC
For transient assay	
35SpNLp1_f	CGGGATCCAAGGAGATATAACAATGGCCCCTTCGCTCTTCC
35SpNLP1_r	CGGAATTCTTAAGCGTAATCTGGAACATCGTATGGGTACAAGGCCGCCTTTCCGAGGTTGTC
H127A_f	GACGGGCCTCGGCGCACGCCACGAGTGGGA
H127A_r	TCCCACTCGTGGCGTGCGCCGAGGCCCGTC
Site-direct mutagenesis for nlp24	
NLP1_XbaI_F	GCGTCTAGACAGACACAATGGCCCCTTCGCTCTTCCGCATCGCC
NLP1_MAMP_R	GGGCATGTACCACGAGGCCGCGGCGGCGTAGCGGCCGTTGAA
NLP1_MAMP_F	TTCAACGGCCGCTACGCCGCCGCGGCCTCGTGGTACATGCCC
TEFpNLP1_r	CCGGAATTCTTAAGCGTAATCTGGAACATCGTATGGGTACAAGGCCGCCTTTCCGAGGTTGTC
C-terminal deletion series of NLP1	
NLP1_XbaI_F	GCGTCTAGACAGACACAATGGCCCCTTCGCTCTTCCGCATCGCC
NLP1_del_177_R	CCGGAATTCTTAAGCGTAATCTGGAACATCGTATGGGTAGTAGCCGACGCGCGGGTGCGTGGAGCC
NLP1_del_197_R	CCGGAATTCTTAAGCGTAATCTGGAACATCGTATGGGTACTGCTGGCCGCCCTGGCCGCTGGTGAA
NLP1_del_217_R	CCGGAATTCTTAAGCGTAATCTGGAACATCGTATGGGTAGTTGGTGTTCTGCAGCGCCGTGCGCGC
Expression of 32 aa C-terminal region	
NLP1_Sp_mCherry_F1	CATACATCACTCTAGACAGACACAATGTACGCCTCAAGCTTC
NLP1_Sp_mCherry_R1	ACCAGAACCACCACCCTTGTACAGCTCGTCCATG
NLP1_217_mCherry_F2	GGTGGTGGTTCTGGTCCCATCCAGCCCCGT
NLP1_32_mCherry_F2	GGTGGTGGTTCTGGTGCCGCGCGCACGGCG
NLP1_Sp_mCherry_R2	GCTTGATATCGAATTCTTACAAGGCCGCCTTTCC
qRT-PCR analysis	
qRT_NLP32_F	GAACACCAACTTTGTCGACGCC
qRT_NLP32_R	AGGTTGTCGTTGAAGTTGCCGT
Co_Act_F	CTCGTTATCGACAATGGTTC
Co_Act_R	GAGTCCTTCTGACCCATACC

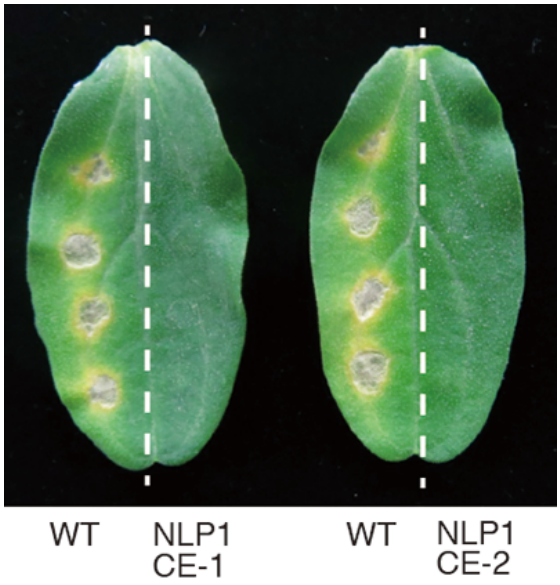

Fig. I-1. Constitutive expression of NLP1 in *C. orbiculare* impaired pathogen infection on cucumber. Inoculation assay of the *C. orbiculare* strains constitutively expressing NLP1 on host cucumber. The wild-type (WT) strain was inoculated onto the left halves of the cucumber cotyledons as a positive control. The test strains were inoculated on the right halves. Inoculated leaves were incubated for 7 days. Among the tested 12 transformants, the 6 transformants failed to develop lesions on cucumber and the result of the two transformants is shown here. WT, The WT strain 104-T; NLP1CE-1 and NLP1CE-2, the two independent strains constitutively expressing NLP1. Similar results were obtained from three independent experiments.

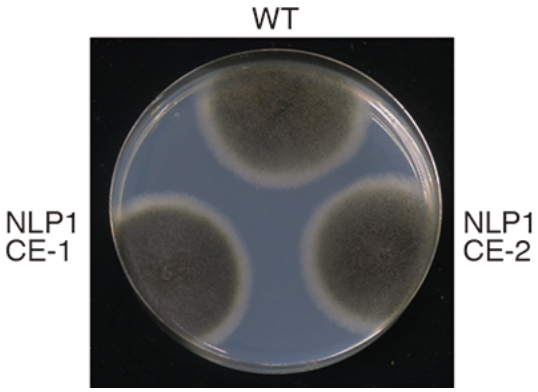

Fig. I-2. Colony phenotype of *C. orbiculare* strains constitutively expressing NLP1. Each stain was grown on potato dextrose agar (PDA) plates for 7 days. WT, the wild-type strain 104-T; NLP1CE-1 and NLP1CE-2, the two independent strains constitutively expressing NLP1.

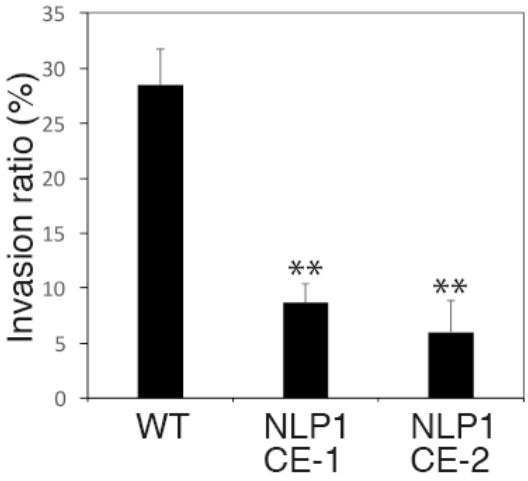

Fig. I-3. Quantitative assay for appressorium-mediated invasion on cucumber. A conidial suspension of each strain was inoculated onto the abaxial surface of cucumber cotyledons and incubated for 4 days. The ratio of appressoria forming invasive hyphae was calculated. In each experiment, at least 150 appressoria were examined and counted to calculate the percentage of invasive hyphae. Means and standard deviations were calculated from three independent experiments. The statistical significance of differences between means was determined by two-tailed t-test. **P < 0.01 (comparison with wild-type).

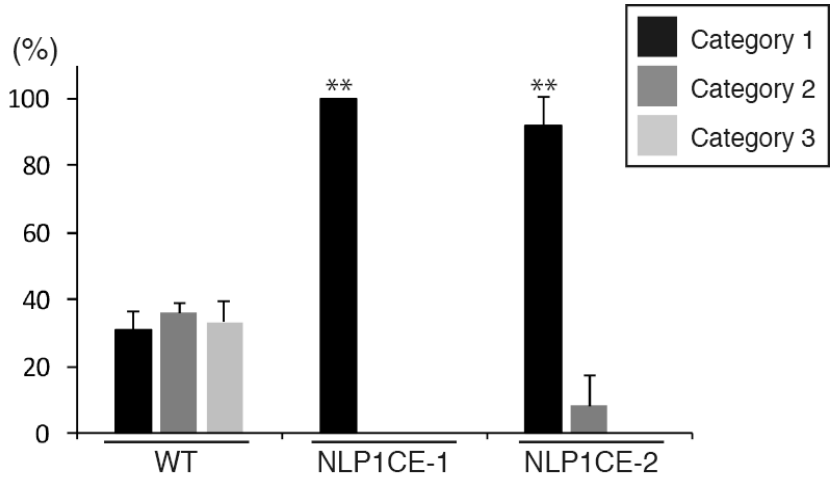

Fig. I-4. Elongation level of primary hyphae formed by appressoria in cucumber epidermis. A conidial suspension of each strain was inoculated onto the abaxial surface of cucumber cotyledons. Inoculated cotyledons were incubated for 4 days and subjected to microscopic observation for primary hyphae formed by appressoria. Elongation of primary invasive hyphae was classified into three categories. Category 1, invasive hypha limited inside a single cell (length < 10 μm); Category 2, invasive hypha limited inside a single cell (length > 10 μm); Category 3, invasive hypha extended to a neighbor cell. In each experiment, at least 150 appressoria were examined for the presence/absence of primary invasive hyphae, and the elongation level of detected invasive hyphae in each strain was classified into the three categories. Means and standard deviations were calculated from three independent experiments. The statistical significance of differences between means of "Category 1" type was determined by two-tailed t-test. **P < 0.01 (comparison with wild-type).

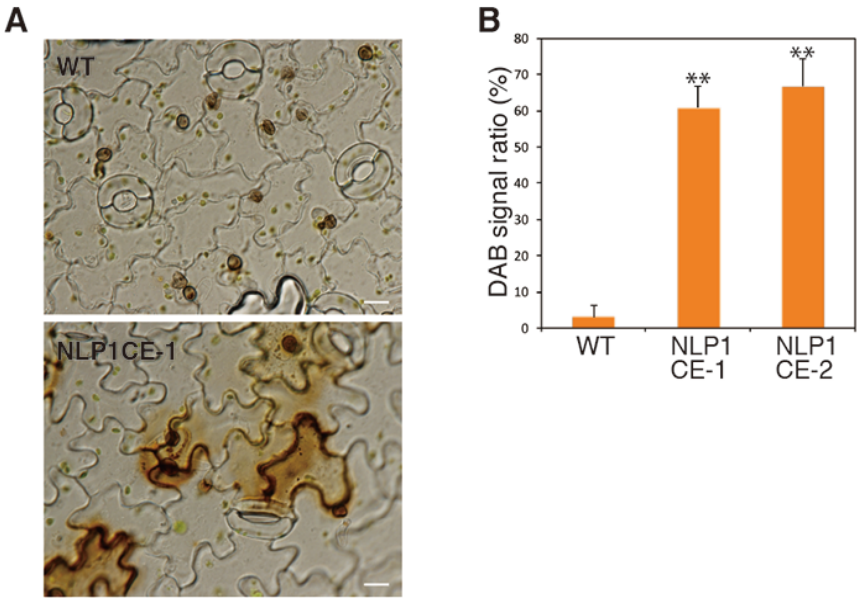

Fig. I-5. NLP1CE strains activate ROS generation in cucumber.

A, 3,3′-diaminobenzidine (DAB) staining assay for the detection of H_2O_2 accumulation. A conidial suspension of each strain was inoculated onto the abaxial surface of cucumber cotyledons and incubated for 2 days. Cotyledons were then stained with DAB and subjected to microscopic observation (note: appressoria do not develop invasive hyphae at this time even in the WT strain). Dark brown staining indicates H_2O_2 accumulation. Similar results were obtained from three independent experiments. Bars = 10 μm.

B, Quantitative analysis for DAB staining. Mean percentage of H_2O_2 accumulation at sites of attempted penetration in each strain. DAB staining ratio was calculated as the mean percentage of appressoria with the penetration pore that displayed brown stained area per total appressoria with the penetration pore. In each experiment, approximately 150 appressoria with the penetration pore were counted for each strain. Means and standard deviations were calculated from three independent experiments. The statistical significance of differences between means was determined by two-tailed t-test. **P < 0.01 (comparison with wild-type).

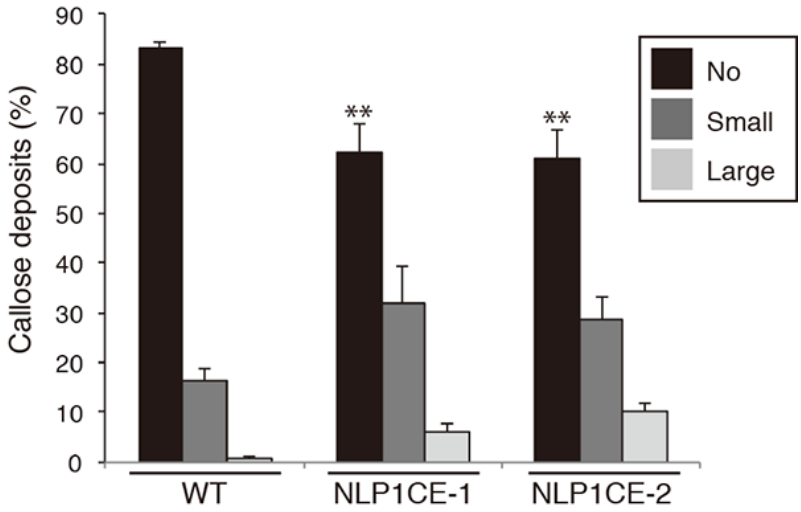

Fig. I-6. Papillary callose deposition assay. Mean percentage of callose deposition formed at sites of attempted penetration by appressoria. Approximately 150 appressoria were counted for each strain. Large: callose formation larger than boundaries of the appressorium; small: callose deposits smaller than boundaries of the appressorium (Fukada et al., 2016). Means and standard deviations were calculated from three independent experiments. The statistical significance of differences between means of "No callose" type was determined by two-tailed t-test. **P < 0.01 (comparison with wild-type).

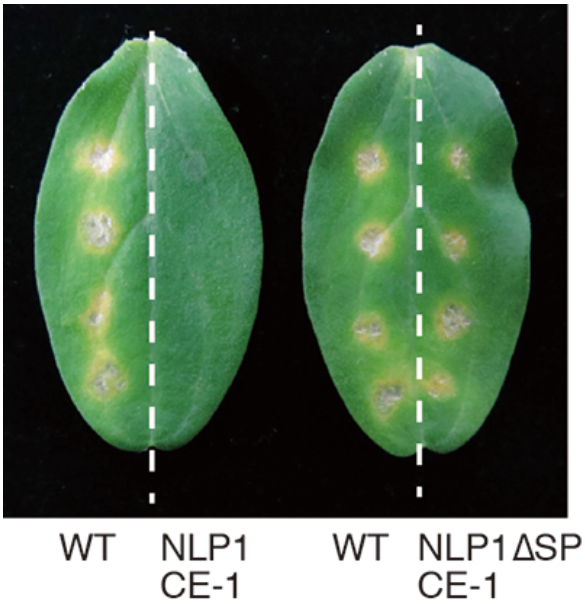

Fig. I-7. Inoculation assay of the strains expressing NLP1 lacking the signal peptide (NLP1ΔSPCE-1). The WT strain 104-T was inoculated onto the left halves as a positive control. On the right halves of the cucumber cotyledons, the test strains were inoculated. Inoculated plants were incubated for 7 days. Similar results were obtained from three independent experiments.

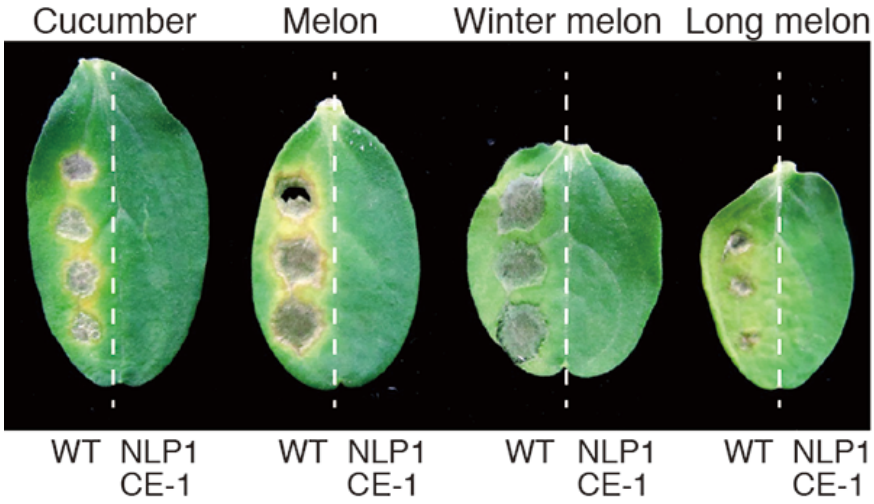

Fig. I-8. Constitutive expression of NLP1 completely blocked the pathogen infection in multiple Cucurbitaceae cultivars. Inoculation assay of the strain NLP1CE-1 on multiple Cucurbitaceae cultivars. The wild-type strain 104-T (WT) was inoculated onto the left halves of the cotyledons derived from each cultivar. NLP1CE-1 was inoculated onto the right halves of the cotyledons. Inoculated leaves were incubated for 7 days. Similar results were obtained from three independent experiments.

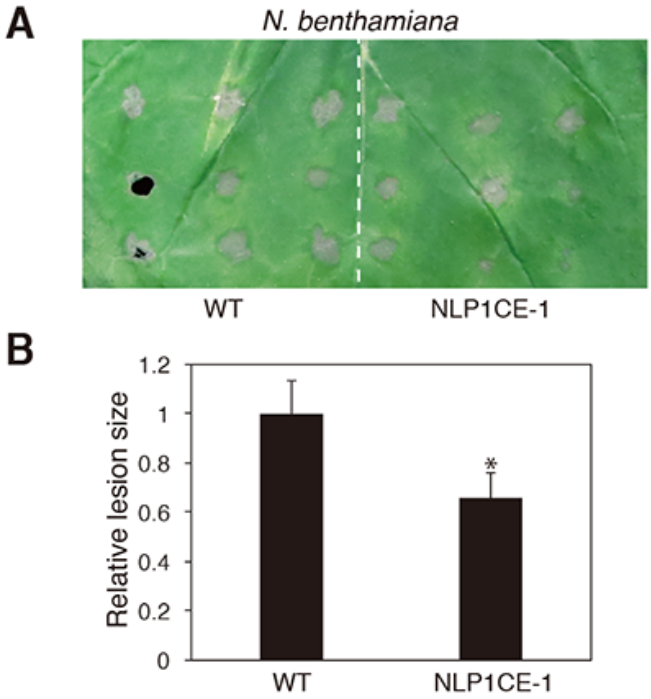

Fig. I-9. Constitutive expression of NLP1 partially inhibited the pathogen infection in *N. benthamiana*.

A, Inoculation assay of the strain NLP1CE-1 on *N. benthamiana*. The WT strain 104-T was inoculated on the left half of *N. benthamiana* leaf. The test strain was inoculated on the right half. Compared with WT, NLP1CE-1 delayed lesion development with a slight reduction of lesion size on *N. benthamiana* leaf. Photographs were taken at 7 days post inoculation (dpi). Similar results were obtained from five independent experiments.

B, Quantitative assay for lesion development area in *N. benthamiana* inoculated by 104-T (WT) and NLP1CE-1. The inoculated leaves were incubated for 7 dpi. For each strain, the total lesion area for 36 inoculated spots was quantified with Image J software. Three independent replicate experiments were performed with similar results. The statistical significance of differences between means was determined by two-tailed t-test. *$P < 0.05$ (comparison with wild-type).

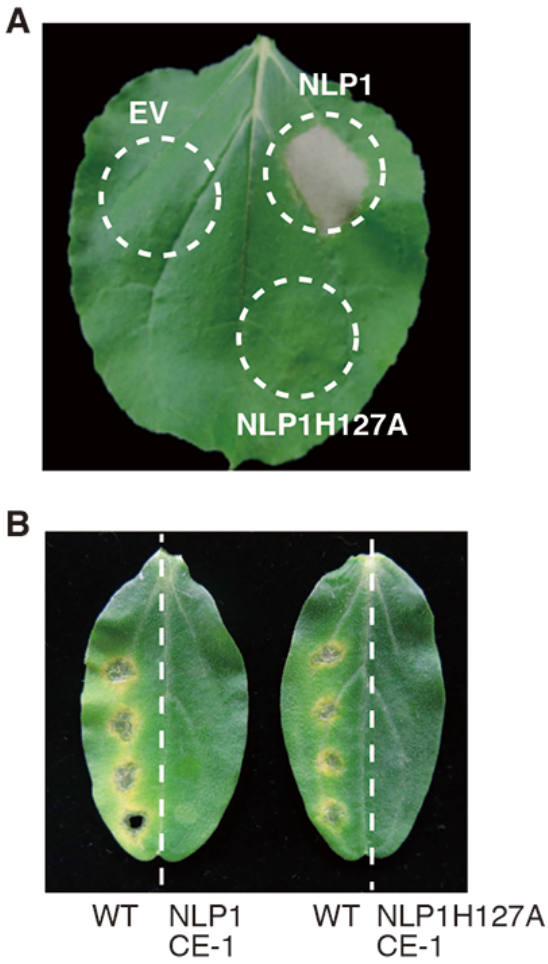

Fig. I-10. NLP1-dependent reduced virulence is uncoupled from its cytotoxic activity.

A, *Agrobacterium*-mediated transient expression of NLP1 and NLP1 lacking the cytotoxic activity in *N. benthamiana* leaves. *A. tumefaciens* carrying empty vector pBICP35 (EV), pBICP35 carrying full-length *NLP1* (NLP1), and pBICP35 carrying *NLP1* having a mutation for cytotoxic activity (NLP1H127A) were infiltrated into *N. benthamiana* leaves. The infiltration sites were represented by dashed circles. The cell death symptoms were assessed at 5 days after the infiltration. Similar results were obtained from three independent experiments.

B, Inoculation of the *C. orbiculare* strain expressing NLP1H127A (NLP1H127ACE-1) on cucumber. The wild-type strain 104-T (WT) was inoculated onto the left halves of cotyledons as a positive control. The test strains were inoculated onto the right halves. The inoculated cotyledons were incubated for 7 days. Similar results were obtained from three independent experiments.

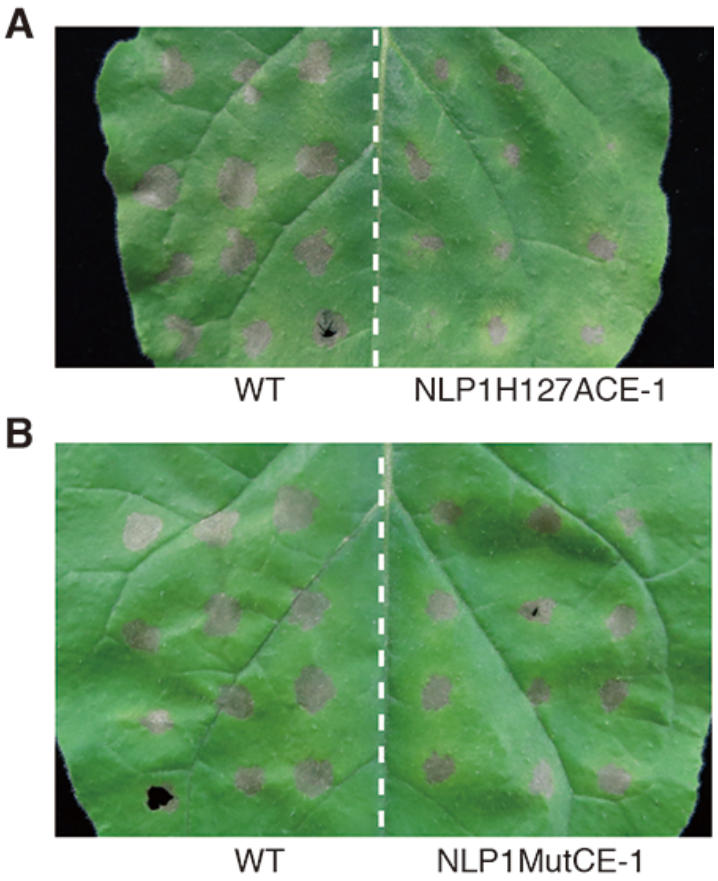

Fig. I-11. Inoculation assay of *C. orbiculare* strains expressing mutated NLPs on *N. benthamiana*. **A,** Inoculation assay of the NLP1H127ACE strain. The inoculated leaves were incubated for 7 days. **B,** Inoculation assay of the NLP1MutCE strain. The inoculated leaves were incubated for 7 days. Compared with the wild-type strain 104-T (WT), both NLP1H127ACE and NLP1MutCE strains slightly reduced the virulence on *N. benthamiana*, which is similar to the case of the NLP1CE strain. Similar results were obtained from three independent experiments.

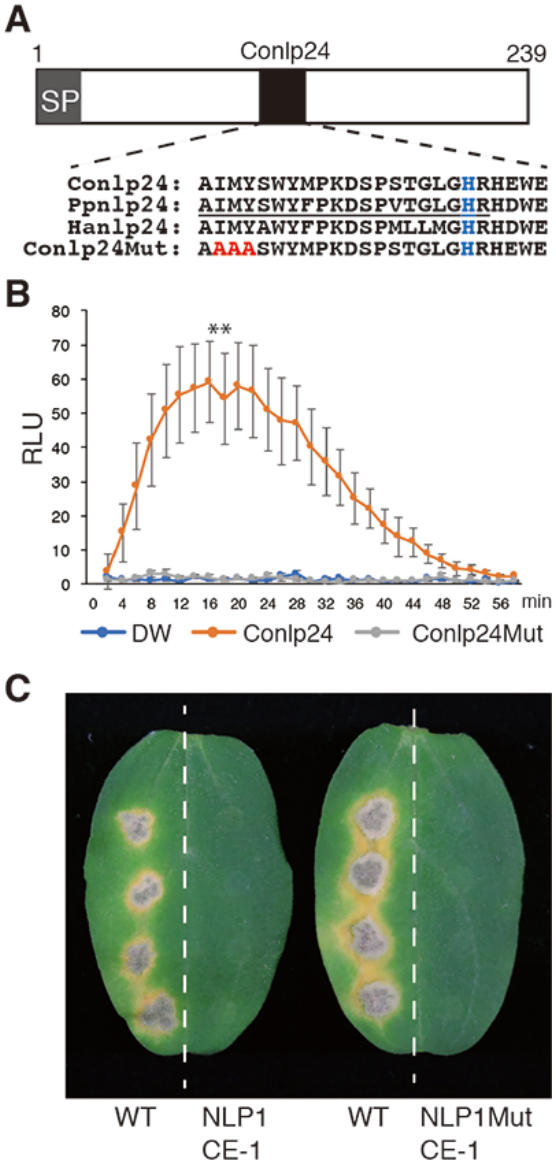

Fig. I-12. The nlp24 sequence of NLP1 is not essential to trigger enhanced defense in cucumber. **A,** Schematic representation of nlp24 in *C. orbiculare* NLP1 with introduced alanine substitutions. nlp24 of *C. orbiculare* (Conlp24) is aligned with nlp24 of *P. parasitica* (Ppnlp24) and of *Hyaloperonospora arabidopsidis* (Hanlp24). Conlp24 with alanine substitutions was also aligned (Conlp24Mut). Mutated amino acids are highlighted by red characters. His127 is highlighted in blue. The Ppnlp20 sequence is underlined. The closed box represents the Conlp24 region. SP represents the signal peptide of NLP1.

B, Reactive oxygen species production induced by 1 μM Conlp24, 1 μM Conlp24Mut, or distilled water (DW) was monitored using a luminol-based assay in leaf discs of *Arabidopsis* Col-0. Data are given as relative light units (RLU) and represent the mean ± SE of 12 wells from one representative experiment of three. The statistical significance of differences between means of total RLU was determined by two-tailed t-test. **P < 0.01 (comparison with DW treatment).

C, Inoculation assay of the strain expressing the mutated NLP1 carrying Conlp24Mut (NLP1MutCE-1) on host cucumber. The wild-type strain 104-T (WT) was inoculated onto the left halves of cucumber cotyledons as a positive control. The test strains were inoculated onto the right halves. The inoculated plants were incubated for 7 days. Similar results were obtained from three independent experiments.

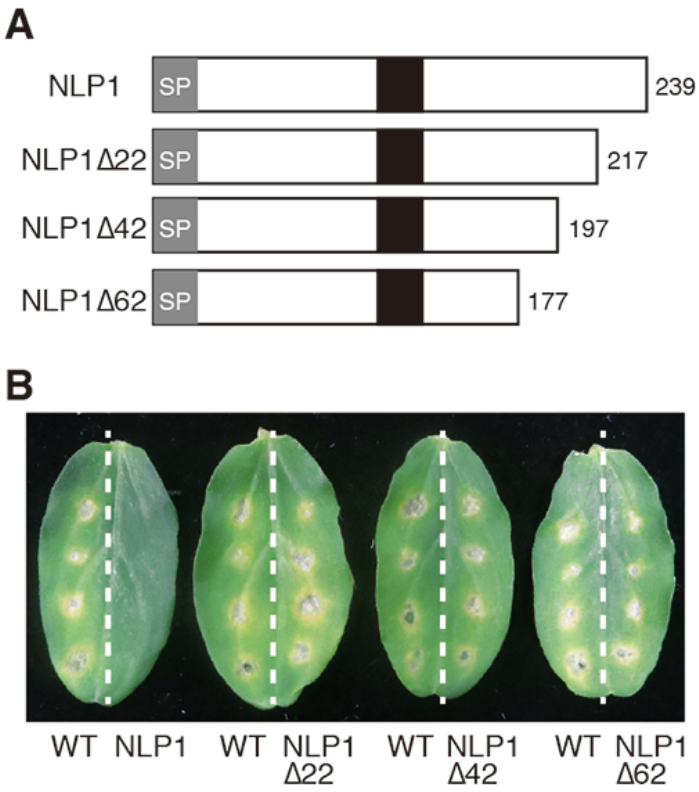

Fig. I-13. The C-terminal region of NLP1 is essential for triggering cucumber defense.

A, Schematic representation of the truncated NLP1 series deleted from its C-terminus. The closed box represents the Conlp24 region. SP represents the signal peptide of NLP1.

B, Inoculation assay of the *C. orbiculare* strains expressing the deleted versions of NLP1. The wild-type strain 104-T (WT) was inoculated onto the left halves of cucumber. On the right halves, the test strains were inoculated. The inoculated cotyledons were incubated for 7 days. Similar results were obtained from three independent experiments.

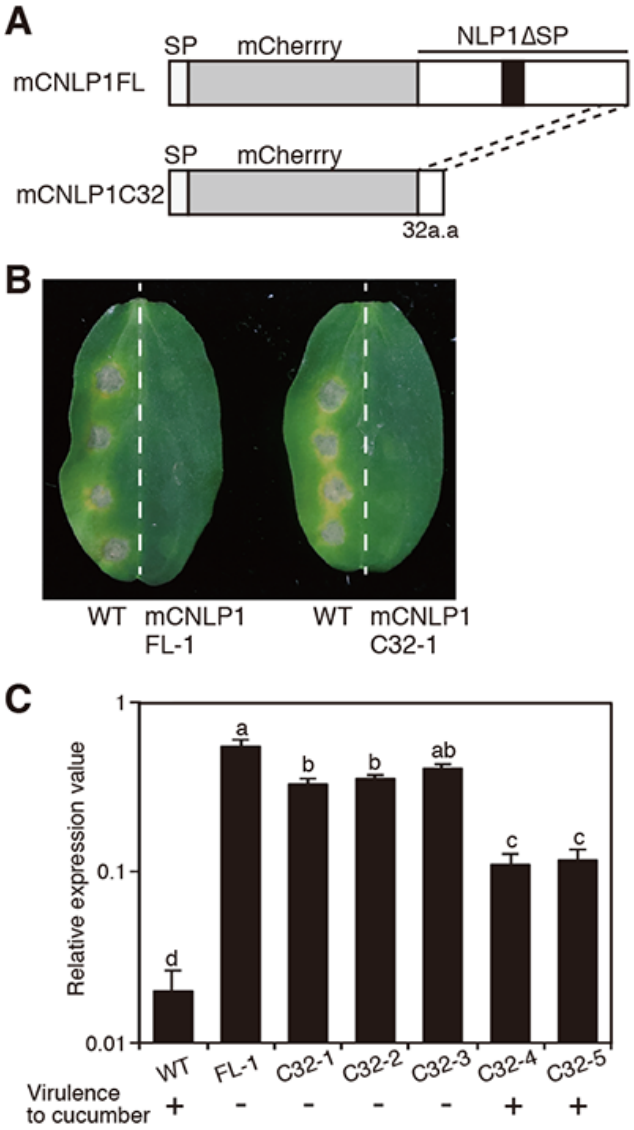

Fig. I-14. Expression of the C-terminal 32 aa of NLP1 as a fusion with SP:mCherry is sufficient to trigger cucumber defense.

A, Schematic diagram for SP:mCherry fused to the NLP1 lacking its signal peptide (mCNLP1FL) or fused to the C-terminal 32 aa of NLP1 (mCNLP1C32). The closed box represents the Conlp24 region. SP represents the signal peptide of CoDN3.

B, Inoculation assay of the strain expressing SP:mCherry fused to the C-terminal 32 aa of NLP1. The wild-type strain 104-T (WT) was inoculated onto the left halves of cucumber cotyledons as a positive control. On the right halves, the test strains (mCNLP1FL-1 and mCNLP1C32-1) were inoculated. The inoculated leaves were incubated for 7 days. Similar results were obtained from three independent experiments.

C, qRT-PCR analysis for expression of the gene encoding SP:mCherry fused to the C-terminal 32 aa of NLP1. Conidial suspensions of tested strains were inoculated on cucumber cotyledons and incubated for 1 day. Means and standard deviations were calculated from three independent experiments. The statistical significance of differences between means was determined on log-transformed data by Tukey test. Means not sharing the same letter are significantly different ($P < 0.05$). FL1, mCNLP1FL-1; C32-1 to C32-5, mCNLP1C32-1 to mCNLP1C32-5. +, WT-like virulence to cucumber; −, no virulence.

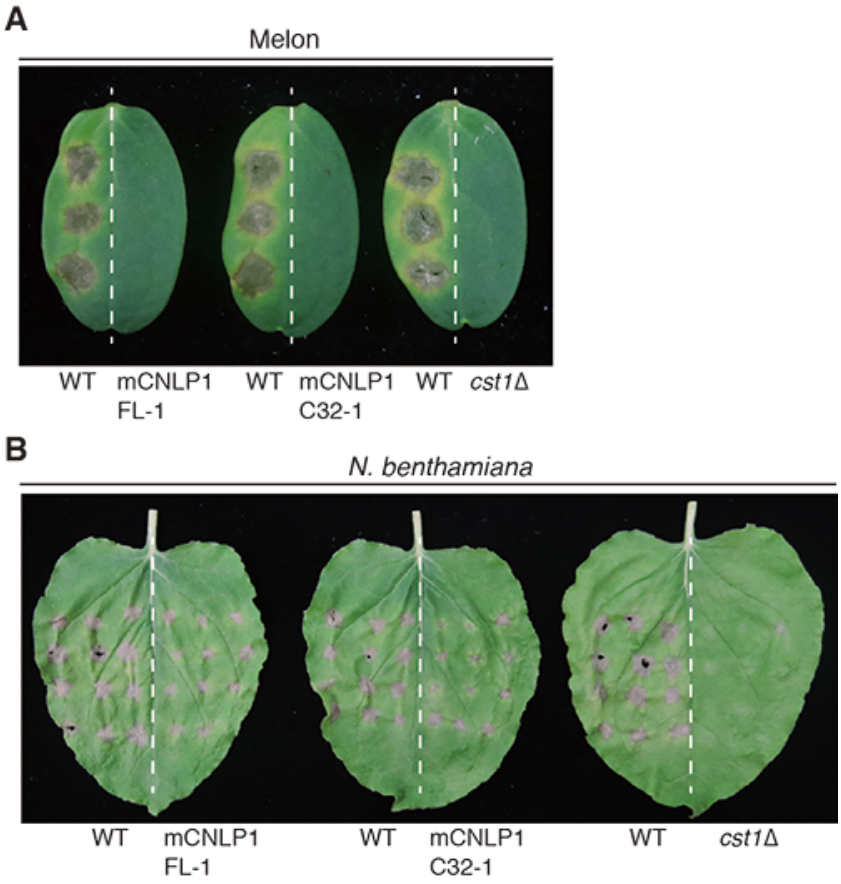

Fig. I-15. The virulence phenotype of the strain mCNLP1C32-1 is identical to that of the strain mCNLP1FL-1 but distinct from that of the *cst1* pathogenicity mutant.

A, Inoculation assay on melon. The *C. orbiculare* strain 104-T (WT) was inoculated on the left halves of cotyledons. The test strains were inoculated on the right halves. The inoculated cotyledons were incubated for 7 days. The strain mCNLP1FL-1 expresses SP:mCherry fused to the NLP1 lacking its signal peptide whereas mCNLP1C32-1 expresses SP:mCherry fused to the C-terminal 32 aa of NLP1. The *cst1* null mutant (*cst1*Δ) is defective in pathogenicity because CST1 is a transcription factor essential for pathogenicity. Similar results were obtained from three independent experiments.

B, Inoculation assay on *N. benthamiana* leaves. Inoculated leaves were incubated for 7 days. The WT developed severe lesions in *N. benthamiana*. Notably, the mCNLP1FL-1 and mCNLP1C32-1 strains developed lesions in *N. benthamiana* although the virulence of these strains were reduced compared with WT. In contrast, the *cst1*Δ mutant failed to develop them. Thus, the C-terminal 32 aa of NLP1 is sufficient to mimic the unique effect of full-length NLP expression in *C. orbiculare* infection on cucumber. Similar results were obtained from three independent experiments.

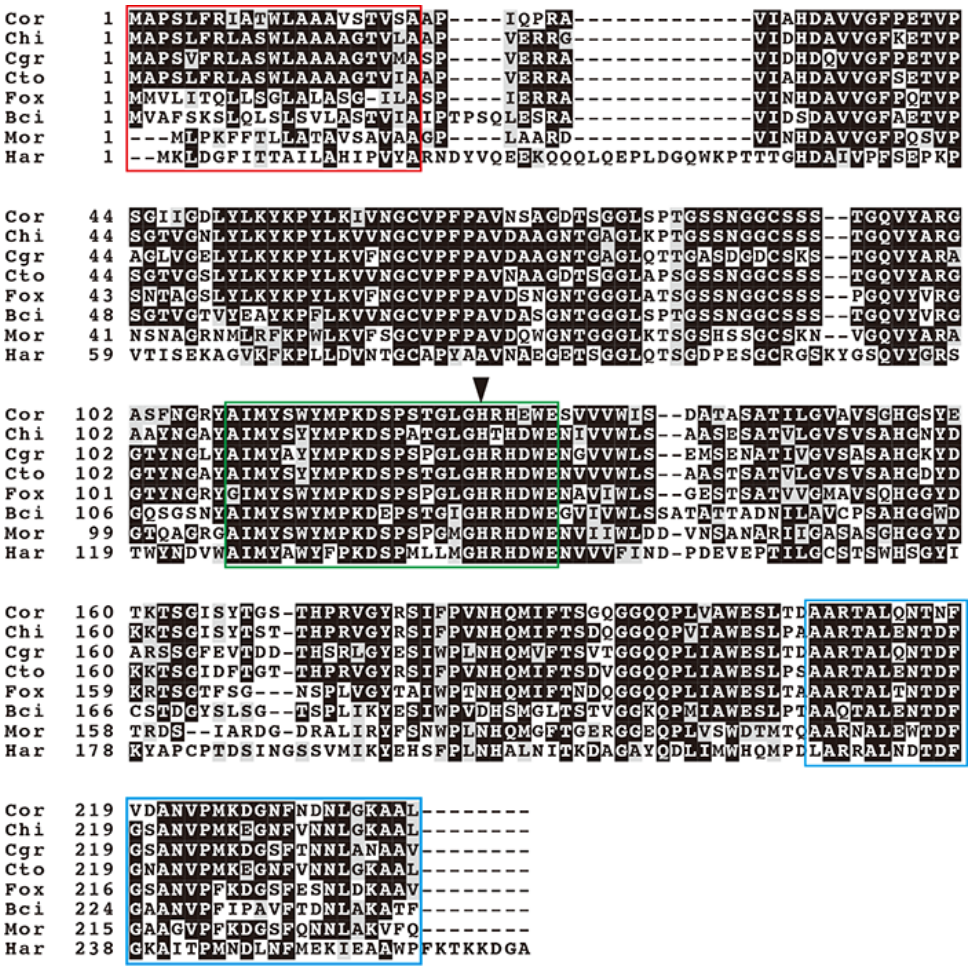

Fig. I-16. Amino acid sequence alignment of *C. orbiculare* NLP1 with its putative homologues in other plant-infecting fungi and oomycete. Sequence data of *C. orbiculare* NLP1 and each NLP1 homologues can be found in the GenBank/EMBL data libraries under the accession numbers ENH78932 (Cob_11657, CoNLP1) for *C. orbiculare*, XP_018154754 for *C. higginsianum*, XP_008099179 for *C. graminicola*, KZL66402 for *C. tofieldiae*, XP_018243470 for *Fusarium oxysporum*, XP_001551049 for *Botrytis cinerea*, XP_003715954 for *Magnaporthe oryzae*, AEZ06577 for *Hyaloperonospora arabidopsidis*. Amino acid sequences were aligned using ClustalW (Thompson et al., 1994). Identical amino acids are indicated as white letters on black background, similar residues are indicated with a gray background, and gaps introduced for alignments are indicated by hyphens. Putative signal peptide sequence was indicated by a red opened box. nlp24 region and C-terminal 32 aa region were indicated by green and blue opened boxes, respectively. His127 is indicated by an arrowhead. Cor, *C. orbiculare*; Chi, *C. higginsianum*; Cgr, *C. graminicola*; Cto, *C. tofieldiae*; Fox, *F. oxysporum*; Bci, *B. cinerea*; Mor, *M. oryzae*; Har, *H. arabidopsidis*.

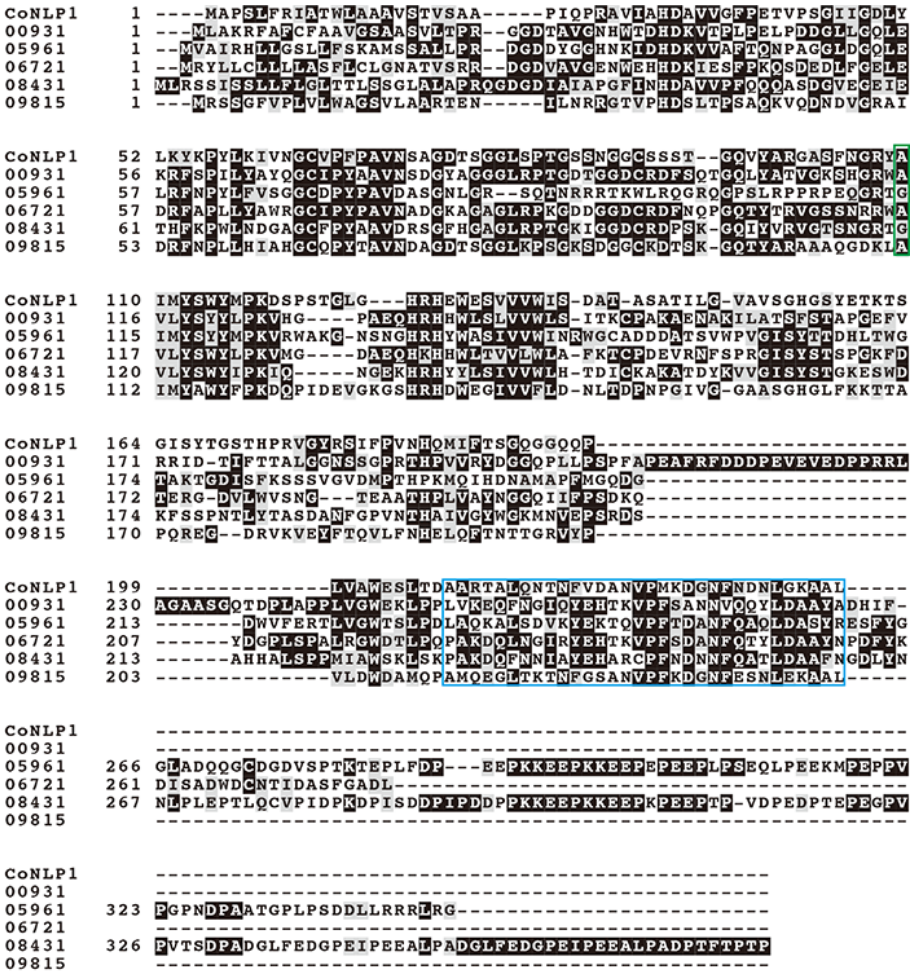

Fig. I-17. Amino acid sequence alignment of *C. orbiculare* NLP1 with other NLPs in *C. orbiculare*. Sequence data of each NLP can be found in the GenBank/EMBL data libraries under the accession numbers ENH81388 (Cob_00931), ENH85690 (Cob_05961), ENH84773 (Cob_06721), ENH82991 (Cob_08431), and ENH81601 (Cob_09815). Amino acid sequences were aligned using ClustalW (Thompson et al., 1994). Identical amino acids are indicated as white letters on black background, similar residues are indicated with a gray background, and gaps introduced for alignments are indicated by hyphens. The region corresponding to the C-terminal 32 aa region was indicated by a blue opened box.

Chapter II

Functional studies of *NLP1* and *NLP1* homologs in *C. orbiculare*

Introduction

In Chapter I, I have demonstrated that the expression of *NLP1* is preferentially expressed in late phase of infection of *C. orbiculare* and have cytotoxic activity in *Nicotiana benthamiana*. Importantly, I found that the constitutive expression of *NLP1* in *C. orbiculare* blocks pathogen infection on Cucurbitaceae cultivars via their defense activation. However, the *NLP1*-dependent inhibition of pathogen infection is not related with its cytotoxic activity or the MAMP sequence that is recognized by *Arabidopsis*. Instead, Cucurbitaceae cultivars recognize the C-terminal region of NLP1 and this recognition strongly activates the Cucurbitaceae defense to terminate *C. orbiculare* infection.

Multiple *NLP* genes from different pathogens have been reported to contribute to pathogen virulence, whereas also multiple *NLP* genes from other species did not seem to affect pathogen virulence. For example, disruption of soft-rot bacterium *Erwinia carotovora* Nip_{ECC} and Nip_{ECA} genes results in reduced virulence on potato (Pemberton et al., 2005). Silencing of *PcNLP2, PcNLP6* and *PcNLP14* in *Phytophtora capsici* also caused reduced virulence of the pathogen on pepper leaves (Feng et al., 2014). Similarly, there is a strong evidence that ectopic expression of *NLP* gene in *Colletotrichum coccodes* accelerated the disease together with enhanced pathogen growth in *Abutilon theophrasti* and furthermore extended its host range (Amsellem et al., 2002). In contrast, targeted deletion of *NLP* genes in *Fusarium oxysporum* f. sp. *erythroxyli*, wheat pathogen *Mycosphaerella graminicola*, and *Botrytis cinerea* did not result in virulence reduction (Bailey et al., 2002; Motteram et al., 2009 and Arenas et al., 2010). Likewise, quadruple

mutants of *MoNLP* in *Magnaporthe oryzae* does not impair the fungal virulence in rice (Fang et al., 2017). These findings implied that the role of NLPs for pathogenic microorganisms during infection is ambiguous. In this chapter, I first asked whether the targeted deletion of the *NLP1* gene affects the virulence of *C. orbiculare* on host plants or not. I also investigated the importance of the C-terminal region of NLP1 for its functionality.

The NLPs have been identified in taxonomically diverse organisms such as bacteria, fungi and oomycetes. All NLP proteins conserve NPP1 domain (Fellbrich et al., 2002) and to date more than 500 NLPs have been identified (Oome and Ackerveken, 2014). In the plant- pathogenic fungus *Mycosphaerella graminicola,* one NLP encoding gene was found (Motteram et al.,2009); while in *Magnaporthe grisea,* four NLP homologues were predicted (Dean et al., 2005). In the pathogenic fungus, *Verticillium dahliae,* eight NLP homologues has been predicted and among them, *VdNEP* was proven to induce necrosis (Zhou et al., 2012). Apart from the cytotoxic NLPs, several NLPs have been found to be unable to induce necrosis. For instance, two out of three NLPs of *Phytophtora infestans* (Kanneganti et al., 2006), 11 out of 19 tested NLPs of *Phytophtora sojae* (Dong et al., 2012), and all NLPs of obligate biotroph oomycete *Hyaloperonospora arabidopsidis* (Cabral et al., 2012) do not cause necrosis when transiently expressed in plants. Most of these noncytotoxic NLPs are expressed at the early phase of infection (Kanneganti et al., 2006; Cabral et al., 2012 and Dong et al., 2012), implying that these proteins may have an alternative role during infection establishment (Oome and Ackerveken, 2014).

It was previously reported that the *C. orbiculare* genome contains six *NLP* gene homologs (Gan et al., 2012). Among them, there are two *NLP* homologs (*Cob_08431* and *Cob_05961*) that exhibit induced expression at early stages of infection. In chapter I, I demonstrated that inappropriate expression of *NLP1* have negative impact on fungal virulence, however, it remains elucidated whether *Cob_08431* and *Cob_05961* give the

same effects or not. In Chapter II, I focused on these two NLP homologs (Cob_08431 and Cob_05961) by investigating their cytotoxic activity and also the effects of their constitutive and strong expression on fungal virulence.

Results

Deletion of the C-terminal region in NLP1 slightly reduced its cytotoxic activity in *Nicotiana benthamiana*

As revealed in Chapter I, the Cucurbits cultivars recognize NLP1 at the C- terminal region of NLP1. Therefore, to further elucidate whether the corresponding C- terminal region is important for the cytotoxic activity of NLP1 or not, I performed transient expression of NLP1Δ22 under the control of 35S promoter in *N. benthamiana*. I found that cell death activity was slightly decreased in the expression of NLP1Δ22 compared to that of the full length NLP1 (Fig. II-1). This result indicates that the 22 amino acids at the C-terminal region is required for full cytotoxic activity of NLP1 but is not essential for the activity. On the other hand, I also performed the transient expression of NLP1 lacking the nlp24 MAMP sequence (NLP1Mut) in *N. benthamiana*. I found that the cell death symptom was greatly reduced in the expression of NLP1Mut compared with the expression of the wild type NLP1 (Fig. II-1).

Deletion of *NLP1* had no detectable effects on *C. orbiculare* virulence

To investigate the function of *NLP1* in *C. orbiculare* virulence, I performed targeted gene replacement of *NLP1* in *C. orbiculare*. I generated the replacement vector

pCB1636NLP1 and introduced it into the wild-type (WT) strain of *C. orbiculare* (Fig. II-2A). By PCR analysis on genome DNA of candidate transformants, I successfully identified two independent *nlp1* null mutants (*nlp1*Δ KO-1 and *nlp1*Δ KO-2) (Fig. II-2B). The *nlp1*Δ strains exhibited similar fungal morphology as the wild-type (WT) strain 104-T grown on potato dextrose agar (PDA) (Fig. II-2C). This suggests that the deletion of *NLP1* did not appear to affect vegetative growth of *C. orbiculare* on PDA.

Next, I drop-inoculated the conidial suspensions of the *nlp1*Δ strains onto detached cucumber cotyledons. I found that the *nlp1*Δ strains retained WT-level virulence on cucumber (Fig. II-3A). Besides cucumber, I also investigated whether the *nlp1*Δ strains of *C. orbiculare* have any effects on the pathogen virulence to other susceptible plants. I performed the pathogenicity assay of the *C. orbiculare nlp1*Δ strains on other susceptible Cucurbitaceae cultivar: melon (*Cucumis melo*) (Fig. II-3B). As a result, we found that the *nlp1*Δ strains developed lesions on melon which is the same as the WT strain. *Nicotiana benthamiana* was also inoculated with the *nlp1*Δ strains and I found that the *nlp1*Δ strains also showed WT-level virulence on the plants (Fig. II-4). Collectively, these results indicate that the deletion of *NLP1* have no detectable effect on *C. orbiculare* virulence on Cucurbitaceae cultivars and *N. benthamiana*.

Functional analysis of Cob_08431

It has been reported that there are 6 *NLP*-like genes including *NLP1* in the *C. orbiculare* genome (Gan et al., 2013). Among them, RNA sequence data indicated that 2 homolog genes, *Cob_08431* and *Cob_05961* are preferentially expressed at early infection phases. *Cob_08431* is preferentially expressed at 3 dpi whereas *Cob_05961* is expressed at 1 dpi. I decided to focus on both *Cob_08431* and *Cob_05961*. First, I generated the transgenic lines of *C. orbiculare* constitutively expressing *Cob_08431*

under the *TEF* promoter (named Cob_08431CE). The Cob_08431CE strains showed the normal colony morphology on PDA the same as WT. I then inoculated the Cob_08431CE strains on detached cucumber cotyledons. In contrast to the strains expressing *NLP1,* the Cob_08431CE strain developed severe lesions in the cucumber cotyledons the same the WT strain (Fig. II-5). Thus, the result suggested that the constitutive expression of *Cob_08431* has no detectable effects on the virulence of *C. orbiculare* toward cucumber. Therefore, I considered that Cob_08431 is unlikely to be recognized by cucumber. The finding is likely consistent with the fact that Cob_08431 is expressed at the early infection stage.

Next, I investigated whether Cob_08431 has activity to induce cell death in *N. benthamiana*. I performed transient expression of *Cob_08431* under cauliflower mosaic virus 35S promoter in *N. benthamiana*. As a result, I found that the transient expression of Cob_08431 slightly induced yellowish symptom on *N. benthamiana* (Fig. II-6). The results suggested that Cob_08431 has cytotoxic activity to some degree but its activity is weaker than that of NLP1.

Functional analysis of Cob_05961

Next, I generated the *C. orbiculare* transgenic lines that constitutively express *Cob_05961* under *TEF* promoter in order to investigate its effect on virulence of the pathogen. However, during the plasmid construction, I found that the amplified *Cob_05961* cDNA sequence showed the presence of 2 additional nucleotides which are adenine and guanine at the end of intron (Fig. II-7), which are absent in the predicted cDNA sequence of *Cob_05961* (Gan et al., 2013). The additional two nucleotides were also confirmed with our RNA-Seq read sample of *C. orbiculare* inoculation on cucumber at 1 dpi. The presence of additional nucleotides caused the frame shift in amino acid

translation, resulting in shorter translated protein that lacks the conserved heptapeptide motif and the known MAMP motif recognized by Arabidopsis (Fig. II-8A and 8B).

This finding raised a possibility that the predicted full-length protein of Cob_05961 has negative effects on virulence of *C. orbiculare*, therefore, the expression of the short and truncated form of Cob_05961 was selected in *C. orbiculare*. To assess this, I generated the *C. orbiculare* transgenic strains that constitutively express *Cob_05961* named as Cob_05961CE strains. I also generated the *C. orbiculare* strains that express the artificial *Cob_05961* lacking A and G nucleotides named as Cob_05961ΔAGCE strains. Subsequently, I inoculated the generated strains on cucumber cotyledons. As a result, I found that both Cob_05961CE and Cob_05961ΔAGCE strains developed severe lesions in the cucumber cotyledons the same as WT strain (Fig. II-9). Thus, unlike the hypothesis, the results suggest that the expression of Cob_05961ΔAG has no negative effects on *C. orbiculare* virulence. Next, in order to investigate whether the original Cob_05961 of *C. orbiculare* or the artificial Cob_05961ΔAG has cytotoxic activity or not, I performed transient expression assay of Cob_05961 and Cob_05961ΔAG under the control of 35S promoter. As a result, no cell death activity was detected when both proteins were expressed on *N. benthamiana* (Figs. II-10 and II-11).

Discussion

The 22 amino acid residues in the C-terminal of NLP1 from *C. orbiculare* has been shown to be recognized by Cucubitaceae cultivars in Chapter I. The agroinfiltration assay showed that NLP1Δ22 still retains cytotoxic activity although NLP1Δ22 slightly reduced its cytotoxic activity compared with the full-length wild type NLP1. Thus this region does not play a major role in inducing cell death on *N. benthamiana*.

In contrast, NLP1Mut completely lost its cytotoxic activity. Consisntently, Ottmann and associates (2009) revealed that several amino acid residues in *Phytium aphanidermatum NLP* (NLP_{Pya}) and a heptapeptide GHRHDWE motif are necessary for plasma membrane permeabilization and cytolysis in plant cells. Studies on *Verticillium dahliae* NLPs conducted by Zhou and associates (2012) also showed that Y112 and several conserved amino acid residues of VdNLP2 in the close vicinity of D119 in the crystal structure ß5 strand were required for necrotic activity of VdNLP2. It is reasonable that *Arabidopsis* recognizes the critical part of NLPs as MAMP sequence, because pathogens need to keep this MAMP sequence if this cytotoxic activity is important for pathogens. NLP1Δ22 might lose unidentified and important function of NLP1 and/or its related homologs in other pathogens.

NLPs are generally considered to contribute to the virulence of necrotrophic fungal and pathogenic bacteria on dicotyledonous plants (Ottmann et al., 2009). For example, work by Pemberton et al., (2005) revealed that the disruption of *Erwinia carotovora* Nip_{ECC} and Nip_{ECA} showed clear reduction in virulence on potato tubers. In addition, the overexpression of Nep1 gene from *Fusarium oxysporum* in *Colletotrichum coccodes* increased its virulence on the weed *Abutilon theophrasti* (Amsellem et al., 2002).

However, the role of NLPs in the fungal virulence remains elusive. The gene disruption analysis of *C. orbiculare NLP1* showed that the mutants were equally virulent as the wild type strain, implying that *NLP1* is not a major pathogenicity factor for this pathogen. This finding is likely in agreement with the observation of the targeted deletion of *NLP* genes in *Mycosphaerella graminicola,* which it did not appear to affect pathogen virulence in wheat (Motteram et al., 2009). Likewise, quadruple mutants of *Magnaporthe oryzae* did not compromise the fungal virulence on rice (Fang et al., 2017). Collectively, these observations provide the idea that NLPs may be dispensable in a part of pathogens including *C. orbiculare* and other components might compensate the absence of NLPs

and mask the potential phenotype of mutant strains (Arenas et al., 2010 and Fang et al., 2017).

Microarray data of *C. orbiculare* suggested that several NLP homologues are expressed at early phases of infection (Gan et al., 2012). Among them, the expression of *Cob_08431* and *Cob_05961* at early infection stages is obvious. In this chapter, I revealed that the constitutive expression of *Cob_08431* and *Cob_05961* in *C. orbiculare* has no detectable effects in virulence of the pathogen, which is distinct from the case of *NLP1*. The finding suggests that Cob_08431 and Cob_05961 are unlikely recognized by cucumber. Consistent with this, the C-terminal regions of Cob_08431 and Cob_05961 exhibit variation to that of NLP1 compared with the NLP1 putative orthologs of other fungi (Figs. I-16 and I-17). I also revealed that Cob_08431 has weak cytotoxic activity whereas Cob_05961 and Cob_05961ΔAG has no detectable activity, suggesting that *C. orbiculare* NLPs expressed in the early infection phase has weak or no cytotoxic activity unlike NLP1 expressed in the necrotrophic phase. Our findings are in agreement with those of Cabral and associates (2012), whose experiments with obligate biotrophic oomycete pathogen *Hyaloperonospora arabidopsidis* showed that *H. arabidopsidis* NLPs expressed during early infection stages did not result in any necrotic response. The finding suggests that these NLPs might contribute to the establishment of biotrophic interactions or any other roles that has no relation with cytotoxic activity. On the other hand, a certain *Phytophtora sojae* NLP with the necrosis inducing activity is expressed at the early phase of infection, thus, this activity may be suppressed by different effectors conveyed to the host by the pathogen (Dong et al., 2012). Further studies are necessary to understand functional diversification of *C. orbiculare* NLPs including noncytotoxic NLPs.

Materials and methods

Fungal strains, media, transformation and DNA analysis

Colletotrichum orbiculare (syn. *C. lagenarium*) wild-type (WT) strain 104-T (MAFF240422) is stored at the Laboratory of Plant Pathology, Kyoto University. All *C. orbiculare* strains were maintained on 3.9% (w/v) potato dextrose agar (PDA; Nissui, Tokyo, Japan) at 24ºC in the dark. The transformation of *C. orbiculare* was based on the method in Kimura et al. (2001). Bialaphos-resistant transformants were selected on minimal medium plates (0.16% yeast nitrogen base without amino acid, 0.2% asparagine, 0.1% NH_4NO_3 and 2% glucose) with 25 μg/mL bialaphos (Wako Pure Chemicals, Japan), meanwhile hygromycin-resistant transformants were selected on PDA plates containing 100 μg/mL hygromycin B (Wako Pure Chemicals, Japan). The total DNA of *C. orbiculare* was isolated from mycelia with the DNeasy plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Plasmid construction

All plasmids used for constitutive expression of NLP1 homologs were constructed using the plasmid pBATTEFP (Asakura et al., 2009). pBATTEFP was generated by introduction of the *TEF* promoter into pBAT (Kimura et al., 2001), a derivative of pCB1531 (Sweigard et al., 1997) that carries the bialaphos resistance gene (*bar*). All primers used in Chapter II are listed in Table II-1. To delete the *NLP1* gene of *C. orbiculare,* a gene disruption vector, pCB1636NLP1, was constructed. The 0.7 kb of 3' flanking region of *NLP1* gene was amplified from the *C. orbiculare* genome with the primers 3NLP1d_for and 3NLP1d_rev. The amplified products were digested with *Xho*I and *Kpn*I and then inserted into the *Xho*I-*Kpn*I site of pCB1636 harboring a hygromycin-resistant gene (*HPH*), to generate pCB1636NLP1_down. The 2.1kb of 5' flanking region

of *NLP1* was amplified with the primers 5NLP1d_for and 5NLP1d_rev. The amplified products were digested with *Xba*I and *Eco*RI and introduced into the *Xba*I-*Eco*RI site of pCB1636, resulting in pCB1636NLP1 replacement vector. To confirm *NLP1* gene disruption, genomic PCR analysis was performed by using two primers, CoNLP1up_for and CoNLP1down_rev containing the border sequences of the *NLP1* gene.

To constitutively express NLP1 homolog *Cob08431* in *C. orbiculare* under the control of the *TEF* promoter, *Cob08431* cDNA fused with an HA epitope tag was amplified by nested PCR. The outer fragment was amplified by primers Cob08431_for and Cob08431_rev in primary PCR. The amplified product of primary PCR was used as a template for the secondary PCR. The secondary PCR was carried out by using the primers TEFpCob08431_f and TEFpCob08431_r. The amplified fragment was digested with *Xba*I and *Eco*RI and introduced into pBATTEFP to produce pBATTEFPCob08431. To constitutively express NLP1 homolog *Cob05961* in *C. orbiculare* under the control of the *TEF* promoter, *Cob05961* cDNA fused with an HA epitope tag was amplified by nested PCR. In the primary PCR, the outer fragment of *Cob05961* was amplified by using primers Cob05961_for and Cob05961_rev. Then, by using the product of primary PCR as a template, the inner region of *Cob05961* was amplified with primers TEFpCob05961_f and TEFpCob05961_r. Next, the amplified fragment was digested with *Xba*I and *Eco*RI and introduced into pBATTEFP to produce pBATTEFPCob05961.

For the construction of mutant *Cob05961* lacking the 2 nucleotides, one single site-directed mutagenesis was carried out to delete the 2 nucleotides which are adenine and guanine in *Cob05961* by recombinant PCR using the primer pairs TEFpCob05961_f/Cob05961dAG_r and Cob05961dAG_f/TEFpCob05961_r with pBATTEFPCob05961 as a template. Then, the full-length fragment of *Cob05961*(ΔAG) fused with an HA epitope tag was amplified using TEFpCob05961_f and TEFpcob05961_r primers. Then, the amplified fragment of the HA-tagged

Cob05961(ΔAG) was digested with *Xba*I and *Eco*RI and then inserted into pBATTEFP, resulting in pBATTEFPCob05961(ΔAG).

To express HA-tagged *Cob08431* under the control of the 35S promoter, a *Bam*HI fragment of *Cob08431* was amplified by using nested PCR. In the primary PCR, the outer fragment was amplified by primers Cob08431_for and Cob08431_rev. The amplified product of primary PCR was used as a template for the secondary PCR. The secondary PCR was carried out by using the primers 35SpCob08431_f and 35SpCob08431_r. Then, the amplified fragment from secondary PCR was digested with *Bam*HI and introduced into the *Bam*HI site of pBICP35, resulting in pBICP35Cob08431. To generate the HA-tagged *Cobo5961* with the 35S promoter, a *Bam*HI-*Eco*RI fragment of *Cob05961* was amplified by using nested PCR. In the primary PCR, the outer fragment of *Cob05961* was amplified by using primers Cob05961_for and Cob05961_rev. Next, by using the product of primary PCR as a template, the inner region of *Cob05961* was amplified with primers 35SpCob05961_f and 35SpCob05961_r. Then, the amplified fragment was digested with *Bam*HI and *Eco*RI and introduced into pBICP35 to produce pBICP35Cob05961.

To express HA-tagged *Cob05961* lacking 2 nucleotides under the control of the 35S promoter, site-directed mutagenesis by primer extension was used to delete the 2 nucleotides which are adenine and guanine by using the primer pairs 35SpCob05961_f/Cob05961_r and Cob05961_f/35SpCob05961_r by using pBICP35Cob05961 as a template. Next, 35SpCob05961_f and 35SpCob05961_r primers were used to amplify the full length of *Cob05961*ΔAG. The amplified fragment was digested with *Bam*HI and *Eco*RI and then inserted into the *Bam*HI–*Eco*RI site of pBICP35, resulting in pBICP35Cob05961ΔAG. To construct HA-tagged *NLP1*Mut and *NLP1*Δ22 under the control of 35S promoter, a *Bam*HI-*Eco*RI fragment of *NLP1*Mut was amplified by using pBATTEFPNLP1Mut as a template. The amplification was carried out by using primers 35SpNLP1_f/35SpNLP1_r. Then, the amplified product was

digested with *Bam*HI and *Eco*RI and then inserted into the *Bam*HI–*Eco*RI site of pBICP35, resulting in pBICP35NLP1Mut. Next, by using pBATTEFPNLP1Δ22 as a template, the *NLP1*Δ22 was amplified by using primers 35SpNLP1_f/35SpNLP1d22_r. The amplified product was then digested by *Bam*HI and *Eco*RI and then inserted into the *Bam*HI–*Eco*RI site of pBICP35, resulting in pBICP35NLP1Δ22.

Inoculation assay of *C. orbiculare*

In the inoculation assay of *C. orbiculare* on cucumber and melon, 10 μL conidial suspensions (5 x 10^5 conidia/mL) were drop-inoculated onto detached cucumber cotyledons (*Cucumis sativus*), melon (*Cucumis melo*). Inoculated cotyledons were incubated at 24ºC for 7 days. The cotyledons were picked from 10 days old plants. For inoculation on *N. benthamiana,* true leaves detached from approximately 5-week-old plants were used. Each virulence test was performed at least three times under the same conditions.

A. tumefaciens*-mediated transient expression in *N. benthamiana

N. benthamiana plants (5 weeks old) were used for the agro-infiltration assay. Plants were grown in a controlled environment chamber at 25ºC with 16 h of illumination per day. Each construct (pBICP35Cob08431, pBICP35Cob05961, pBICP35Cob05961ΔAG, pBICP35NLP1Mut and pBICP35NLP1Δ22) was transformed into *Agrobacterium* strain GV3101 by electroporation. Each transformant was cultured in YEP containing kanamycin (50 μg/mL), rifampicin (50 μg/mL) and gentamycin (50 μg/mL). The cells were harvested by centrifugation and were suspended in MMA induction buffer (1 L of MMA, 5 g of Murashige-Skoog salts, 1.95 g of MES, 20 g of sucrose and 200 μM acetosyringone, pH 5.6) (Yoshino et al., 2012). All suspensions were incubated for 1–3 h at room temperature prior to infiltration. The suspensions were infiltrated into *N.*

benthamiana leaves using a syringe. The infiltrated areas were observed at 5 days after the infiltration.

Table II-1. Primers used in study in Chapter II.

Name	Sequence
5NLP1d_for	CCGCTCTAGAACTAGTGCGTGCAGTGCGTGTACGTGCG
5NLP1d_rev	GCTTGATATCGAATTCTGTCTGTGTGAAGGACCTTTTG
3NLP1d_for	GTCGACCTCGAGCAGATGAGGATGATGACGTACG
3NLP1d_rev	GGTACCCTGGTGCGACGCGCTTAAATCG
CoNLP1up_for	CCAGCGTCGCTCGTGGTCGTGACAAGT
CoNLP1down_rev	TGAAACCATTCTCAAGCCCAACTCGGC
Cob08431_for	ACTTGAGCGTGTGTGTCTTCTACC
Cob08431_rev	CCGCTGAAACTCCCAAGTGGTTGA
TEFpCob08431_f	GCTCTAGACAGACACAATGTTGCGTTCTTCGATATCGTCGCTC
TEFpCob08431_r	CCGGAATTCCTAAGCGTAATCTGGAACATCGTATGGGTAGGGAGTTGGC GTAAAGGTAGGGTC
Cob05961_for	TTCAAGCCGCTTCACGCCCGTTGC
Cob05961_rev	TCTGCAACAGTACCGAGATGTCAA
TEFpCob05961_f	GCTCTAGACAGACACAATGGTGGCCATTCGTCACCTCCTGGGC
TEFpCob05961_r	CCGGAATTCTTAAGCGTAATCTGGAACATCGTATGGGTAACCACGCAGTC TTCGTCTCAACAG
Cob05961dAG_r	TTGGTCTGAGACCGCCCGAGGTTGCCGCTG
Cob05961dAG_f	CAGCGGCAACCTCGGGCGGTCTCAGACCAA
35SpCob08431_f	CGGGATCCAAGGAGATATAACAATGTTGCGTTCTTCGATATCGTCGCTC
35SpCob08431_r	CGGGATCCCTAAGCGTAATCTGGAACATCGTATGGGTAGGGAGTTGGCG TAAAGGTAGGGTC
35SpCob05961_r	CGGAATTCTTAAGCGTAATCTGGAACATCGTATGGGTAACCACGCAGTCT TCGTCTCAACAG
35SpCob05961_f	CGGGATCCAAGGAGATATAACAATGGTGGCCATTCGTCACCTCCTGGGC
Cob05961_rt_f	ATGGTGGCCATTCGTCACCTCCT
35SpNLP1d22_r	CGGAATTCTTAAGCGTAATCTGGAACATCGTATGGGTAGTTGGTGTTCTG CAGCGCCGTGCGCGC
35SpNLP1_f	CGGGATCCAAGGAGATATAACAATGGCCCCTTCGCTCTTCC
35SpNLP1_r	CGGAATTCTTAAGCGTAATCTGGAACATCGTATGGGTACAAGGCCGCCTT TCCGAGGTTGTC

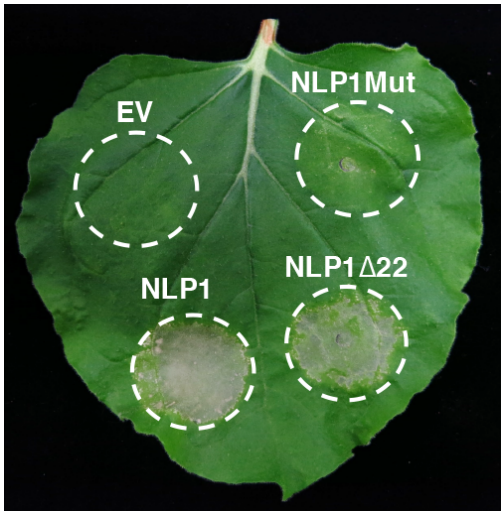

Fig. II-1. NLP1Δ22 induced cell death in *N. benthamiana*. Agrobacterium-mediated transient expression of NLP1, NLP1Mut and NLP1Δ22 in *N. benthamiana* leaves. *A. tumefaciens* carrying empty vector pBICP35 (EV), or *A. tumefaciens* carrying a plasmid expressing full-length NLP1 (NLP1), NLP1Mut or NLP1Δ22 were infiltrated into *N. benthamiana* leaves. The infiltration sites were represented by dashed circles. The cell death symptoms were assessed at 5 days after the infiltration.

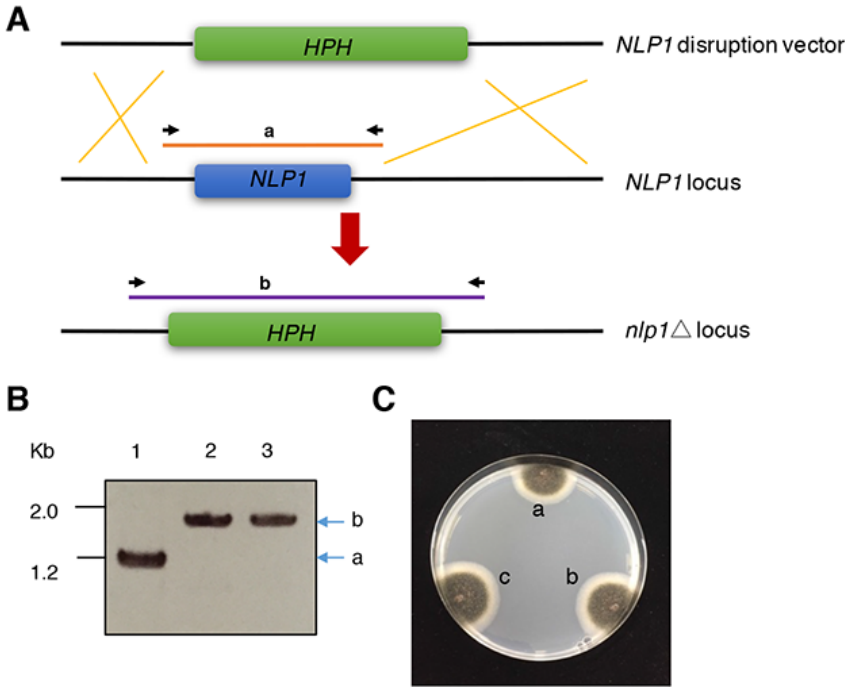

Fig. II-2. Targeted gene disruption of *NLP1*.

A, *NLP1* locus of *C. orbiculare* and *NLP1* disruption vector containing a hygromycin phosphotransferase gene (*HPH*) cassette flanked by the border sequences of *NLP1*. The primers used for the genomic PCR are indicated by arrows. The fragments amplified by PCR are indicated by bars (a and b).

B, Genomic PCR analysis of the *NLP1* gene disruption mutants. Genomic DNAs were isolated from the wild-type strains 104-T or strains transformed with *NLP1* gene disruption vector. A 1.2 kb fragment (a) was amplified from the wild-ype strain (lane 1) whereas a 1.9 kb fragments (b) was amplified from the *nlp1*Δ KO strains (lane 2 and lane 3).

C, Colony phenotype of the *nlp1*Δ KO strains. The wild-type strain 104-T (a) and the *nlp1*Δ strains, *nlp1*Δ KO-1 (b) and *nlp1*Δ KO-2 (c), were incubated on PDA at 24˚C for 7 days.

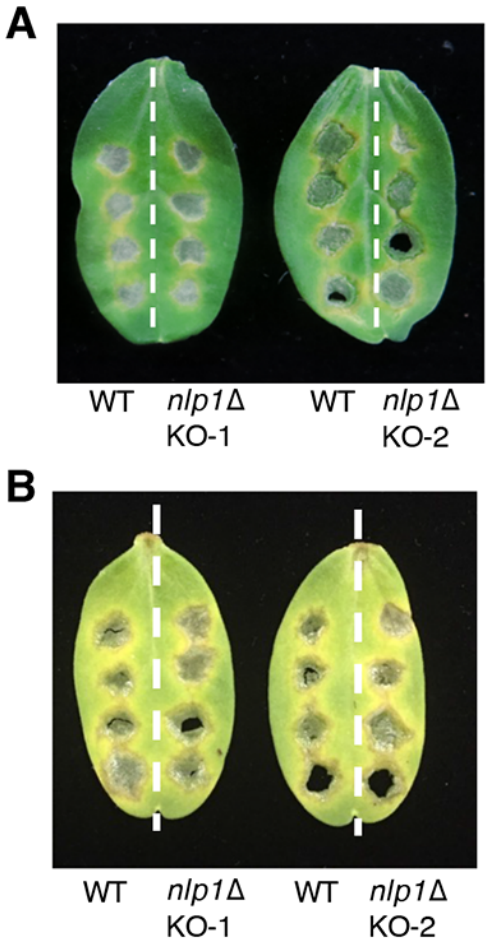

Fig. II-3. Deletion of *NLP1* gives no visible effects on virulence of *C. orbiculare*.

A, Inoculation assay of the *nlp1*Δ strains on cucumber. The wild-type strain was inoculated onto the left halves of the cucumber cotyledons as a positive control. The test strains were inoculated on the right halves. Inoculated leaves were incubated for 7 days at 24˚C. WT, the wild type strain; *nlp1*Δ KO-1 and *nlp1*Δ KO-2, the two independent *nlp1*Δ strains.

B, Conidial suspensions of tested strains were spotted onto melon cotyledons. On the left halves of the cotyledons, the wild-type strain was inoculated as a positive control. On the right halves, the *nlp1*Δ 1KO-1 or the *nlp1*Δ KO-2 was inoculated. The inoculated cotyledons were incubated at 24˚C for 7 days.

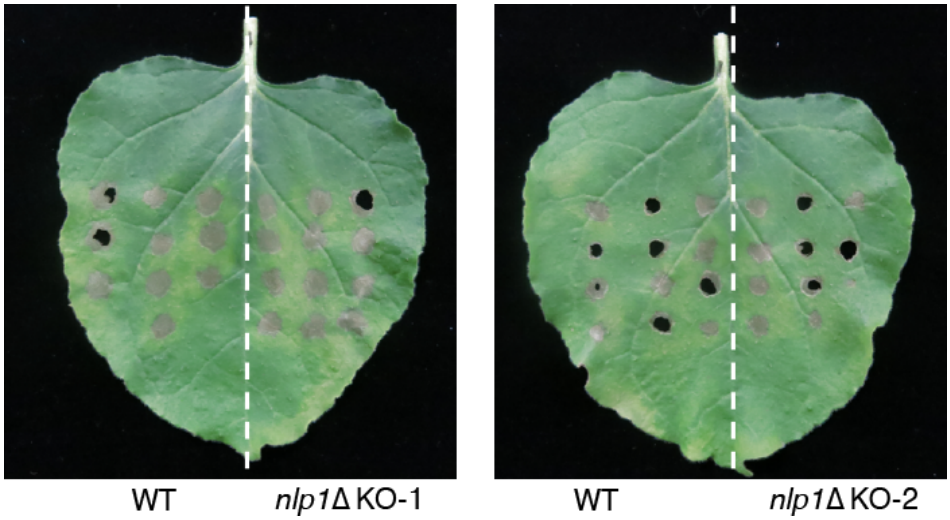

Fig. II-4. *NLP1* is not essential for fungal virulence toward *N. benthamiana*. Inoculation assay of the *nlp1*Δ strain on *N. benthamiana*. The WT strain 104-T was inoculated on the left halves of *N. benthamiana* leaves. The test strains were inoculated on the right halves. The inoculated leaves were incubated at 24˚C for 7 days. The *nlp1*Δ strains developed lesions similar to the wild-type strain on *N. benthamiana*.

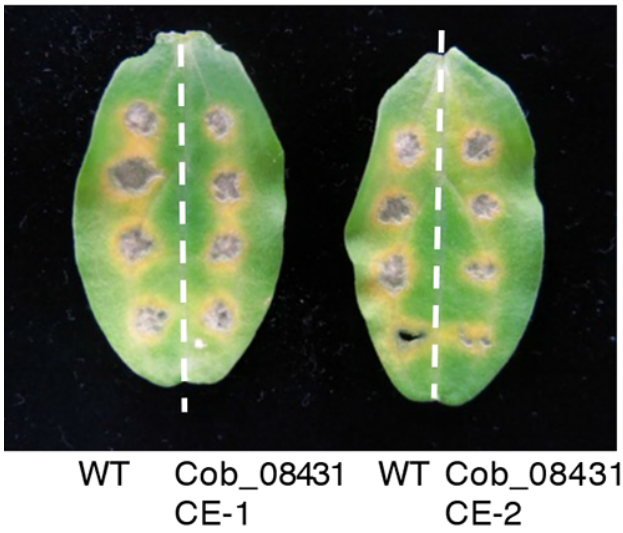

Fig. II-5. Constitutive expression of *Cob_08431* in *C. orbiculare* has no effects on pathogen virulence on cucumber. Conidial suspensions of *C. orbiculare* strains constitutively expressing *Cob_08431* were drop-inoculated on the right halves of the cucumber cotyledons. On the left halves of the cotyledons, the wild-type strain 104-T was inoculated as a positive control. The cotyledons were incubated for 7 days at 24˚C. WT, 104-T; Cob_08431CE-1 and Cob_08431CE-2, the two independent strains constitutively expressing *Cob_08431*.

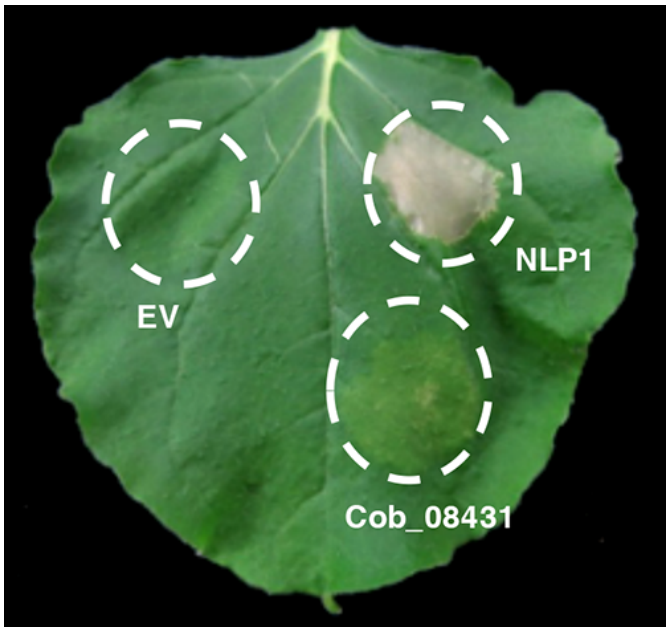

Fig. II-6. Transient expression of Cob_08431 slightly induced cell death in *N. benthamiana*. Agrobacterium-mediated transient expression of NLP1 and Cob_08431 in *N. benthamiana* leaves. *A. tumefaciens* carrying empty vector pBICP35 (EV), or *A. tumefaciens* carrying a plasmid expressing full-length NLP1 (NLP1) or Cob_08431 were infiltrated into *N. benthamiana* leaves. The infiltration sites were represented by dashed circles. The cell death symptoms were assessed at 5 days after the infiltration.

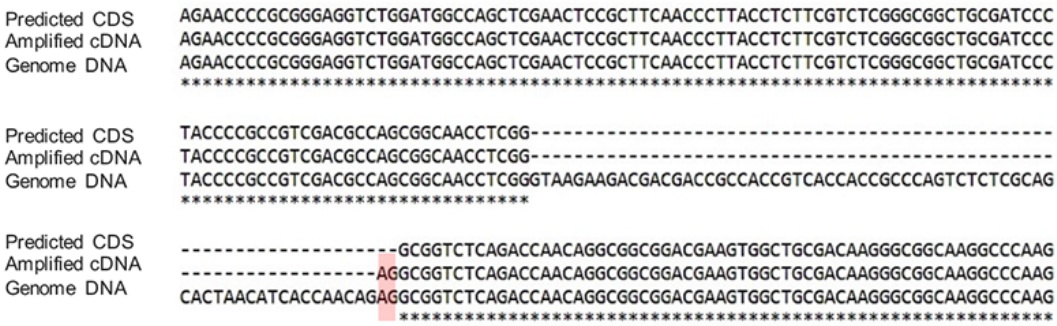

Fig. II-7. Nucleotide sequence alignment of the predicted CDS, the amplified cDNA, and the genome DNA of *Cob_05961*. I aligned (i) CDS based on *C. orbiculare* genome project database (Predicted CDS), (ii) the sequence of *Cob_05961* cDNA amplified from WT cDNA pools prepared at 1 dpi on cucumber (Amplified cDNA) and (iii) genome DNA of *Cob_05961* from *C. orbiculare* genome project database (Genome DNA). The sequence alignment revealed the presence of 2 additional nucleotides, adenine (A) and guanine (G), highlighted with red in Amplified cDNA but not in Predicted CDS after the position of the intron.

A

MVAIRHLLGSLLFSKAMSSALLPRDGDDYGGHNKIDHDKVVAFTQNPAGGLDGQLELRF
NPYLFVSGGCDPYPAVDASGNLGRSQTNRRRTKWLRQGRQGPSLRPPRPEQGRTGIMYS
YYMPKVRWAKGNSNGHRHYWASIVVWINRWGCADDDATSVWPVGISYTTDHLTWGTAKT
GDISFKSSSVGVDMPTHPKMQIHDNAMAPFMGQDGDWVFERTLVGWTSLPDLAQKALSD
VKYEKTQVPFTDANFQAQLDASYRESFYGGLADQQGCDGDVSPTKTEPLFDPEEPKKEE
PKKEEPEPEEPLPSEQLPEEKMPEPPVPGPNDPAATGPLPSDDLLRRRLRG

B

MVAIRHLLGSLLFSKAMSSALLPRDGDDYGGHNKIDHDKVVAFTQNPAGGLDGQLELRF
NPYLFVSGGCDPYPAVDASGNLEGGLRPTGGGRSGCDKGGKAQVYARRGQSRAAPASCT
ATTCPRSAGPRATATATGTTGPASSSGSTAGAAPTTTPPPSGPWASPTRRTT

Fig. II-8. The deduced amino acid sequence encoded by *Cob_05961* and *Cob_05961* lacking two nucleotides A and G.

A, Deduced amino acid sequences encoded by the predicted *Cob_05961* gene based on the prediction software by Gan et al., 2013. *Cob_05961* encodes a protein of 347 amino acids. The conserved heptapeptide motif is present and indicated by red.

B, The presence of 2 additional nucleotides in the *Cob_05961* transcript caused the frame shift, resulting the short and truncated Cob_05961 lacking the conserved heptapeptide motif.

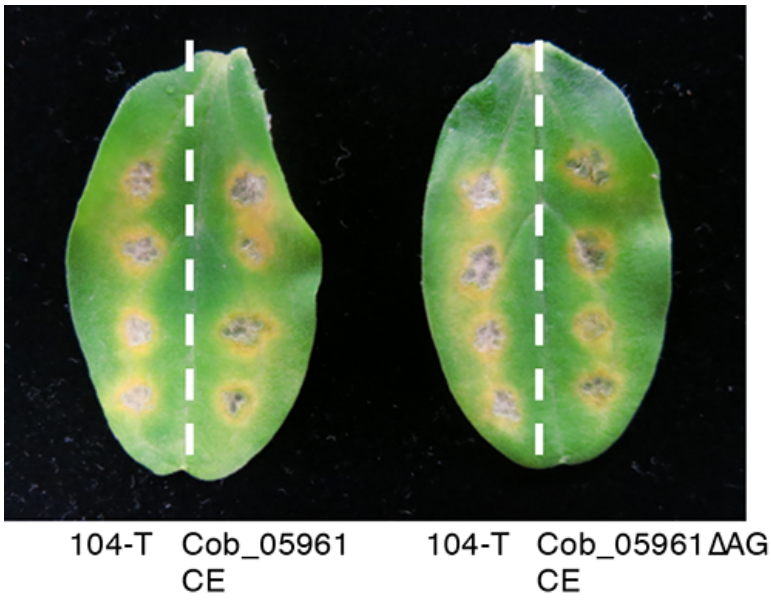

Fig. II-9. Overexpression of Cob_05961 in *C. orbiculare* does not have any effects in its virulence on cucumber. Inoculation assay of the strain Cob_05961CE and the strain Cob_05961ΔAGCE on cucumber cotyledons. The wild-type strain 104-T (WT) was inoculated onto the left halves of the cotyledons as the positive control. On the right halves of the cucumber cotyledons, the test strains were inoculated. Inoculated leaves were incubated for 7 days at 24°C.

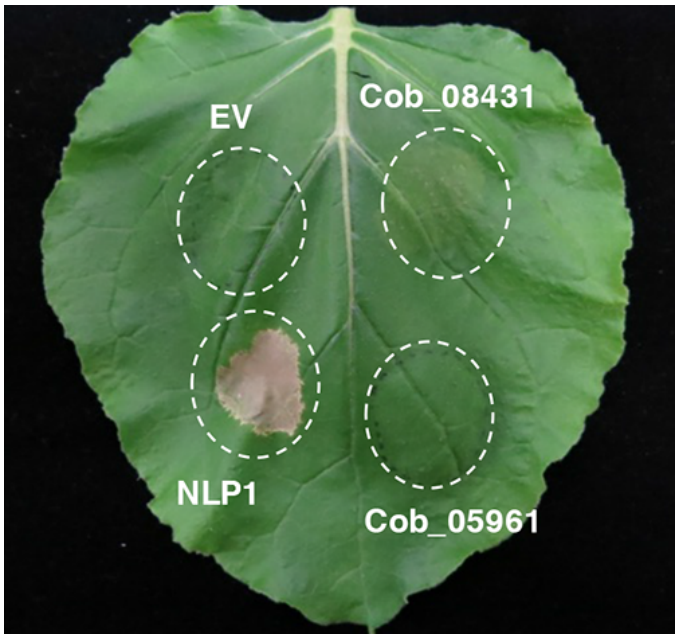

Fig. II-10. The expression of Cob_05961 did not induce cell death in *N. benthamiana*. *A. tumefaciens* carrying the empty vector pBICP35 (EV), or *A. tumefaciens* carrying a plasmid expressing full-length NLP1 (NLP1), Cob_08431 or Cob_05961 were infiltrated into *N. benthamiana* leaves. The infiltration sites were represented by dashed circles. The cell death symptoms were assessed at 5 days after the infiltration.

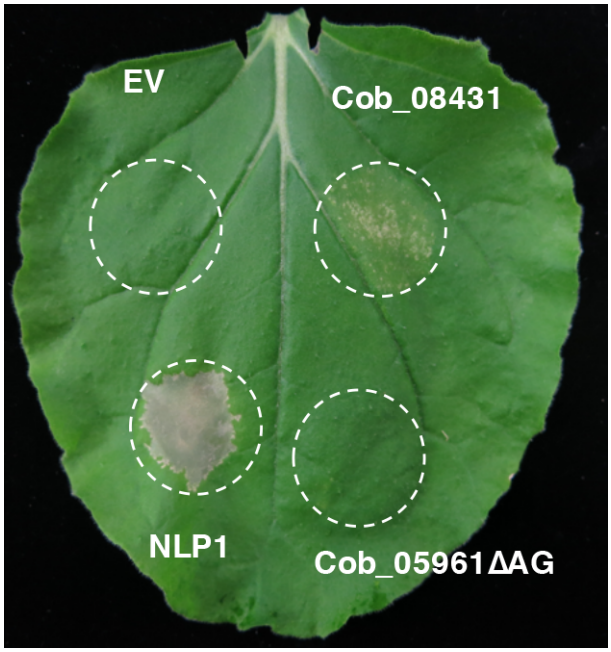

Fig. II-11. The expression of Cob_05961ΔAG did not induce cell death in *N. benthamiana*. *A. tumefaciens* carrying the empty vector pBICP35 (EV), or *A. tumefaciens* carrying a plasmid expressing full-length NLP1 (NLP1), Cob_08431 or Cob_05961ΔAG were infiltrated into *N. benthamiana* leaves. The infiltration sites were represented by dashed circles. The cell death symptoms were assessed at 5 days after the infiltration.

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Summary

Chapter I.

A hemibiotrophic pathogen *Colletotrichum orbiculare* preferentially expresses a necrosis and ethylene-inducing peptide 1 (Nep1)-like protein named NLP1 during the switch to necrotrophy. I here revealed that the constitutive expression of NLP1 in *C. orbiculare* blocks pathogen infection in multiple Cucurbitaceae cultivars via their enhanced defense responses such as ROS generation and callose deposition. NLP1 has a cytotoxic activity that induces cell death in *Nicotiana benthamiana*. However, *C. orbiculare* transgenic lines constitutively expressing a mutant NLP1 lacking the cytotoxic activity still failed to infect cucumber, indicating no clear relationship between cytotoxic activity and the NLP1-dependent enhanced defense in cucurbits. NLP1 also possesses the microbe-associated molecular pattern (MAMP) sequence called nlp24 recognized by *Arabidopsis thaliana* at its central region similar to other pathogens' NLPs. Surprisingly, inappropriate expression of a mutant NLP1 lacking the MAMP signature is also effective for blocking pathogen infection, uncoupling the infection block from the corresponding MAMP. Notably, the deletion analyses of NLP1 suggested that the C-terminal region of NLP1 is critical to enhance defense in cucumber. The expression of mCherry fused with the C-terminal 32 amino acids of NLP1 was enough to trigger the defense of cucurbits, revealing that the C-terminal region of the NLP1 protein is recognized by cucurbits, and then terminates *C. orbiculare* infection.

Chapter II.

The NLP family is broadly conserved among microorganisms including bacteria, fungi and oomycetes. In Chapter I, I revealed that the 22 amino acid residues at C- terminal of *C. orbiculare* NLP1 is recognized by Cucurbitaceae cultivars, whereas the central region of NLP1 (nlp24) is recognized as MAMP by Arabidopsis. In order to gain further understanding on two MAMP sequences, I performed the transient expression of NLP1 lacking 22 amino acids of C- terminal region (NLP1Δ22) or NLP1 lacking nlp24 sequence (NLP1Mut) in *N. benthamiana*. NLP1Mut largely reduced the cytotoxic activity in *N. benthamiana* whereas NLP1Δ22 slightly reduced the activity, indicating the importance of the two MAMP sequence for the cytotoxic activity is distinct. To gain insights on the role of NLP1 in fungal virulence, I generated the targeted deletion mutants for *NLP1*. I show that the *nlp1* null mutants exhibit virulence on host plants the same as the wild-type strain. I further studied the *C. orbiculare NLP* homologs (*Cob_08431* and *Cob_05961*) expressed at the early phase of infection in contrast to *NLP1* expressed at the necrotrophic phase. I revealed that the overexpression of *Cob_08431* and *Cob_05961* in *C. orbiculare* did not give negative effects on virulence of the pathogen on cucumber, suggesting that these NLPs expressed at the early infection phase are unlikely recognized by cucurbits. In addition, I found that Cob_08431 and Cob_05961 have no strong activity to induce cell death in *N. benthamiana.* These findings suggest that Cob_08431 and Cob_05961 might have unidentified roles for the early infection phase, that is not related to cytotoxicity.

Acknowledgments

First and foremost, I am deeply indebted to Prof. Dr. Yoshitaka Takano, my supervisor, whose selfless time and care were sometimes all that kept me going. He provided me with every bit of patient guidance, encouragement, assistance, and expertise throughout my time as his student. I remain astonished that despite his busy schedule, he supported me greatly and was always willing to help me. I also remain extremely grateful for his understanding and support during the times when I was really down and depressed due to personal problems. He has shown me, by his example what a respectable scientist, teacher and person should be.

I would like to express my gratitude to Professor Dr. Ryohei Terauchi (Kyoto University) and Professor Dr. Chihiro Tanaka (Kyoto University) for kindly reviewing this thesis.

Very special thanks to Dr. Suthitar Singkaravanit Ogawa, who worked actively to provide me with her support, guidance throughout my study and her willingness to proof read my thesis draft. Completing this work would have been more tough were it is not for her support.

I must express my gratitude to Assoc. Prof. Dr. Kazuyuki Mise and Dr. Masanori Kaido for their valuable suggestions for my work. I would also like to thank Mrs. Kyoko Ikeda, Dr. Saeko Tanaka and Dr. Yoshihiro Inoue for their technical assistance, helpful discussions during the course of my study.

A special thanks to the really supportive friends, in particular, Ayumi Kosaka and Zhang Ru, not only for all their useful suggestions but also for being there to listen when I needed an ear.

A big ‘Thank You!’ also goes to Mrs. Keiko Hashimoto and all the previous and present members of the Laboratory of Plant Pathology, Kyoto University who made my life experience in Kyoto enjoyable and unforgettable.

Words cannot describe my thankfulness towards my parents; Mr. Ahmad Azmi Ahmad Nordin and Mrs. Zaitul Azma Zainon Hamzah and my mother in law; Mrs Mariam Ismail for their constant unconditional support.

Most importantly, I wish to thank my loving and supportive husband, Rushduddin Abdul Razaq Sowell and my son, Nuh Abdul Rahman Sowell who provide endless emotional supports.

Lastly, I thank the Ministry of Higher Education of Malaysia and International Islamic University Malaysia which provided me with a scholarship for my Doctoral study.