Comparative transient expression analyses on two conserved effectors of Colletotrichum orbiculare reveal their distinct cell death‐inducing activities between Nicotiana benthamiana and melon

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Comparative transient expression analyses on two conserved effectors of *Colletotrichum orbiculare* reveal their distinct cell death-inducing activities between *Nicotiana benthamiana* and melon

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Abstract

Colletotrichum orbiculare infects cucurbits, such as cucumber and melon (*Cucumis melo*), as well as the model Solanaceae plant *Nicotiana benthamiana*, by secreting an arsenal of effectors that suppress the immunity of these distinct plants. Two conserved effectors of *C. orbiculare*, called NLP1 and NIS1, induce cell death responses in *N. benthamiana*, but it is unclear whether they exhibit the same activity in Cucurbitaceae plants. In this study, we established a new *Agrobacterium*-mediated transient expression system to investigate the cell death-inducing activity of NLP1 and NIS1 in melon. NLP1 strongly induced cell death in melon but, in contrast to the effects seen in *N. benthamiana*, mutations either in the heptapeptide motif or in the putative glycosylinositol phosphorylceramide-binding site did not cancel its cell death-inducing activity in melon. Furthermore, NLP1 lacking the signal peptide caused cell death in melon but not in *N. benthamiana*. Study of the transient expression of NIS1 also revealed that, unlike in *N. benthamiana*, NIS1 did not induce cell death in melon. In contrast, NIS1 suppressed flg22-induced reactive oxygen species generation in melon, as seen in *N. benthamiana*. These findings indicate distinct cell death-inducing activities of NLP1 and NIS1 in these two plant species that *C. orbiculare* infects.

KEYWORDS

cell death, *Colletotrichum*, cucurbits, effectors, transient expression

Anthracnose fungi belonging to the genus *Colletotrichum* cause severe diseases in a wide range of crop and ornamental plants. Most *Colletotrichum* species take a hemibiotrophic infection strategy, that is, the pathogen initially grows biotrophically in living host cells and then switches to a destructive necrotrophic phase of infection (Perfect et al., 1999). In the biotrophic stage, it is thought that *Colletotrichum* fungi synthesize and secrete an arsenal of effectors that suppress host immunity and sustain the viability of host tissues. In the subsequent necrotrophic stage, it is assumed that the pathogen secretes toxins, lytic enzymes, and other types of effectors to

kill host cells and gains nutrients to support fungal growth in planta (Gan et al., 2013; Kleemann et al., 2012; O'Connell et al., 2012). *Colletotrichum orbiculare* causes anthracnose disease in cucurbits such as cucumber and melon, but interestingly, the pathogen can also infect *Nicotiana benthamiana*, which is distantly related to cucurbits (Shen et al., 2001; Takano et al., 2006).

Some effectors are widely distributed across species (Bart et al., 2012; Irieda et al., 2019; Liu et al., 2019). One typical example is necrosis- and ethylene-inducing-like proteins (NLPs), which are conserved in many pathogenic bacteria, fungi, and oomycetes (Gijzen & Nürnberger, 2006). Many NLPs are cytotoxic proteins with a strong ability to induce necrosis in eudicot plants such as *N. benthamiana* via binding to eudicot plant-specific sphingolipids called glycosylinositol phosphorylceramides (GIPCs) (Lenarčič et al., 2017; Pemberton & Salmond, 2004). The conserved NLP domain, which has the heptapeptide motif GHRHDWE present in its central region, is required for the cell death-inducing activity (Ottmann et al., 2009; Seidl & Van den Ackerveken, 2019). We recently reported that *C. orbiculare* expresses a typical NLP protein, named NLP1, in the late infection phase and that the *Agrobacterium*-mediated transient expression of NLP1 induces necrosis in *N. benthamiana* (Azmi et al., 2018). However, it is unclear whether NLP1 can induce cell death in host Cucurbitaceae plants that *C. orbiculare* naturally infects.

In this study, we investigated the cell death-inducing activity of NLP1 and various NLP1 mutants in melon (*Cucumis melo*). For this purpose, we established a new *Agrobacterium*-mediated transient expression system in melon using a pEAQ-HT vector designed to allow quick and efficient production of recombinant proteins in plants (Sainsbury et al., 2009). We transformed pEAQ-HT (empty vector [EV]) and pEAQ-HT-GFP into *Agrobacterium tumefaciens* GV3101 (pMP90) by electroporation (Koncz & Schell, 1986). The suspension of transformed *Agrobacterium* was infiltrated (hereafter called agroinfiltration) into melon cotyledons from the abaxial surface using a needleless syringe. We found that MES-KOH, included in the infiltration buffer, was toxic to melon cotyledons and produced insufficient green fluorescent protein (GFP) production, whereas removal of MES-KOH from the infiltration buffer significantly improved GFP expression in the melon cotyledon (Figure S1). The GFP production increased further when we changed to *Agrobacterium* strain GV2260 transformed with pBBRgabT (hereafter called GV2260 [gabT]; Nonaka et al., 2017) instead of GV3101 (pMP90) (Figure S2). Therefore, we decided to use *Agrobacterium* GV2260 (gabT) suspended in the infiltration buffer without MES-KOH for the agroinfiltration assay.

We next infiltrated *Agrobacterium* GV2260 (gabT) with pEAQ-HT-NLP1:HA (primers used for plasmid construction are listed in Table S1) into melon cotyledons and *N. benthamiana* leaves. The infiltrated areas were observed at 5 days postinfiltration. For melon cotyledons, we infiltrated the suspension into the whole area of cotyledons. The transient expression of NLP1 induced necrotic symptoms in both *N. benthamiana* leaves (Figure 1b,c) and melon cotyledons (Figure 2a,b). In melon cotyledons, on average, 15.6% of the area of cotyledons infiltrated with *Agrobacterium* carrying pEAQ-HT-NLP1:HA displayed yellowish or necrotic symptoms, whereas cotyledons infiltrated with *Agrobacterium* carrying pEAQ-HT (EV) did not exhibit such symptoms. These results show that NLP1 exhibits cell death-inducing activity toward two unrelated plant species (*N. benthamiana* and melon) that *C. orbiculare* is able to infect.

Given the tertiary structure of the NLP of *Pythium aphanidermatum* (NLP_{Pya}), three amino acid residues, H101, D104, and E106, from the central heptapeptide motif GHRHDWE and its associated conserved residue D93 are thought to be involved in the coordination of a Mg^{2+} ion within the negatively charged cavity exposed at the protein surface (Figure 1a; Ottmann et al., 2009). Supporting this idea, single mutations of D93, H101, and E106 to alanine (D93A, H101A, and E106A, respectively), in two NLP_{Pya} homologs, NLP_{Pp} from *Phytophthora parasitica* and NLP_{Pcc} from *Pectobacterium carotovorum*, resulted in the loss of ability to trigger necrosis in *N. benthamiana* leaves. These results confirmed that these amino acid residues are required for the biological activity of NLP tested in *N. benthamiana* (Ottmann et al., 2009). Consistently, H127A in the *C. orbiculare* NLP1, which corresponds to H101A in NLP_{Pya}, diminishes cell death-inducing activity in *N. benthamiana* (Azmi et al., 2018; Ottmann et al., 2009). To determine whether this mutation also impairs the activity of NLP1 in melon, we constructed pEAQ-HT-$NLP1^{H127A}$:HA to express NLP1:HA carrying H127A (hereafter called $NLP1^{H127A}$) in melon (Figure 1a). Using this new system, we confirmed that, unlike the wild-type (WT) NLP1, the expression of $NLP1^{H127A}$ did not result in the development of necrotic lesions in *N. benthamiana* (Figure 1b,c). Surprisingly, in melon, the expression of $NLP1^{H127A}$ resulted in yellowish and necrotic symptoms, similar to those observed in the WT NLP1 (Figure 2a,b). Western blotting using an anti-HA antibody showed that NLP1 and $NLP1^{H127A}$ proteins accumulated in melon cotyledons at similar levels (Figure 2c). These findings suggest that H127 of *C. orbiculare* NLP1 is essential for the cell death-inducing activity in *N. benthamiana*, but not in melon.

To extend this finding, we also investigated the effect of triple mutation D119A/H127A/E132A (corresponding to D93A/H101A/E106A in NLP_{Pya}) on the cell death-inducing activity of NLP1 in both melon and *N. benthamiana* (Figure 1a). For this purpose, we constructed pEAQ-HT-$NLP1^{D119A/H127A/E132A}$:HA (Table S1) and introduced it into the *Agrobacterium* strain. An agroinfiltration assay revealed that $NLP1^{D119A/H127A/E132A}$ caused yellowish and necrotic symptoms in melon, as seen in WT NLP1 (Figure 2a,b). This finding suggests that these amino acids, located in the central heptapeptide motif GHRHDWE or its neighbour region, are dispensable for the cell death-inducing activity of NLP1 in melon. Interestingly, in *N. benthamiana*, $NLP1^{D119A/H127A/E132A}$ induced necrotic lesions to some extent, whereas $NLP1^{H127A}$ did not induce any necrotic lesions (Figure 1b,c).

A recent report has shown that the surface-exposed GIPCs in eudicot plants are NLP_{Pya} toxin receptors that bind to NLP_{Pya} H101 and D158, causing a conformational change to facilitate the insertion of NLP_{Pya} into the plasma membrane of *N. benthamiana* (Lenarčič et al., 2017). NLP_{Pya} W155A failed to bind to GIPC and exhibited no cytotoxic activity toward *N. benthamiana*, suggesting that the

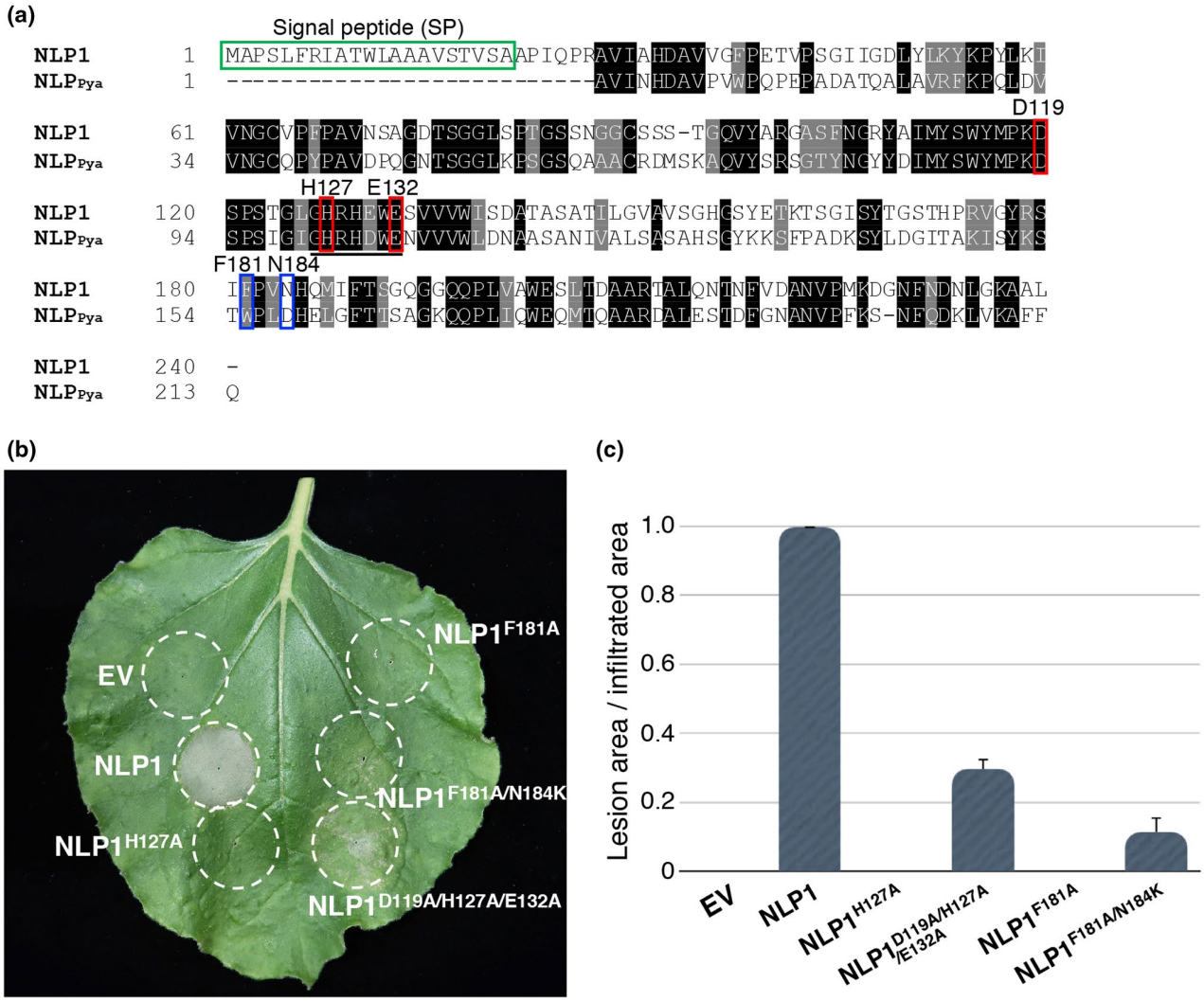

FIGURE 1 Cell death-inducing activity of wild-type and mutated NLP1 proteins expressed in *Nicotiana benthamiana*. (a) The amino acid sequences of *Colletotrichum orbiculare* NLP1 and NLP_{Pya}. NLP1 (GenBank: TDZ25257.1) was aligned with the amino acid sequence of NLP_{Pya} (GenBank: 3GNU_P) using ClustalW (Thompson et al., 1994). Amino acids of NLP_{Pya} in the red box are located within the heptapeptide motif (underlined) and thought to be involved in coordination of the Mg^{2+} ion. We performed mutational analyses of the corresponding amino acids of NLP1 (D119, H127, and E132). Amino acids in the blue box of NLP_{Pya} are involved in binding glycosylinositol phosphorylceramides (GIPCs) in the eudicot plant cell membrane. We also performed mutational analyses of the corresponding amino acids of NLP1 (F181 and N184). The amino acid sequence in the green box is the signal peptide (SP) sequence of NLP1 predicted by SignalP 5.0 (http://www.cbs.dtu.dk/services/SignalP/). We used the amino acid sequence of NLP_{Pya} lacking the N-terminal region for the alignment because the crystal structure of the corresponding region and subsequent mutational analyses have been reported (Ottmann et al., 2009). (b) Transient expression assay of the wild-type and mutated NLP1 proteins in *N. benthamiana*. *N. benthamiana* was challenged with *Agrobacterium tumefaciens* GV2260 (gabT) harbouring pEAQ-HT (empty vector [EV]), pEAQ-HT-NLP1:HA, pEAQ-HT-$NLP1^{H127A}$:HA, pEAQ-HT-$NLP1^{D119A/H127A/E132A}$:HA, pEAQ-HT-$NLP1^{F181A}$:HA, or pEAQ-HT-$NLP1^{F181A/N184K}$:HA. The photograph was taken at 5 days postinfiltration. Similar results were obtained in two additional experiments. (c) Quantitative analysis of the lesion area per infiltration area. Means and standard errors were calculated from three independent experiments. Amino acid abbreviations: A, alanine; D, aspartic acid; E, glutamic acid; F, phenylalanine; H, histidine; K, lysine; N, asparagine

hydrophobic residue W155 near D158, the GIPC-binding site, is also involved in the interaction with plant cell membrane. It has also been shown that both NLP_{Pya} W155A and NLP_{Pya} D158K severely reduce the cytotoxic activity toward two Brassicaceae plants, *Arabidopsis thaliana* and *Brassica oleracea* (Lenarčič et al., 2017). We next asked whether the GIPC-binding ability of NLP1 is related to its cytotoxic activity in melon and *N. benthamiana*. The amino acid alignment analysis of NLP1 and NLP_{Pya} suggested that the corresponding residue of W155 in NLP_{Pya} is F181 in NLP1 (Figure 1a), which is consistent with the fact that both amino acids are aromatic and hydrophobic amino acids.

We constructed pEAQ-HT-$NLP1^{F181A}$:HA (Table S1) and introduced it into the *Agrobacterium* strain. We also constructed pEAQ-HT-$NLP1^{F181A/N184K}$:HA (N184 in NLP1 corresponds to D158 in NLP_{Pya}) (Figure 1a) and then generated another *Agrobacterium* strain harbouring this plasmid. The agroinfiltration assay revealed

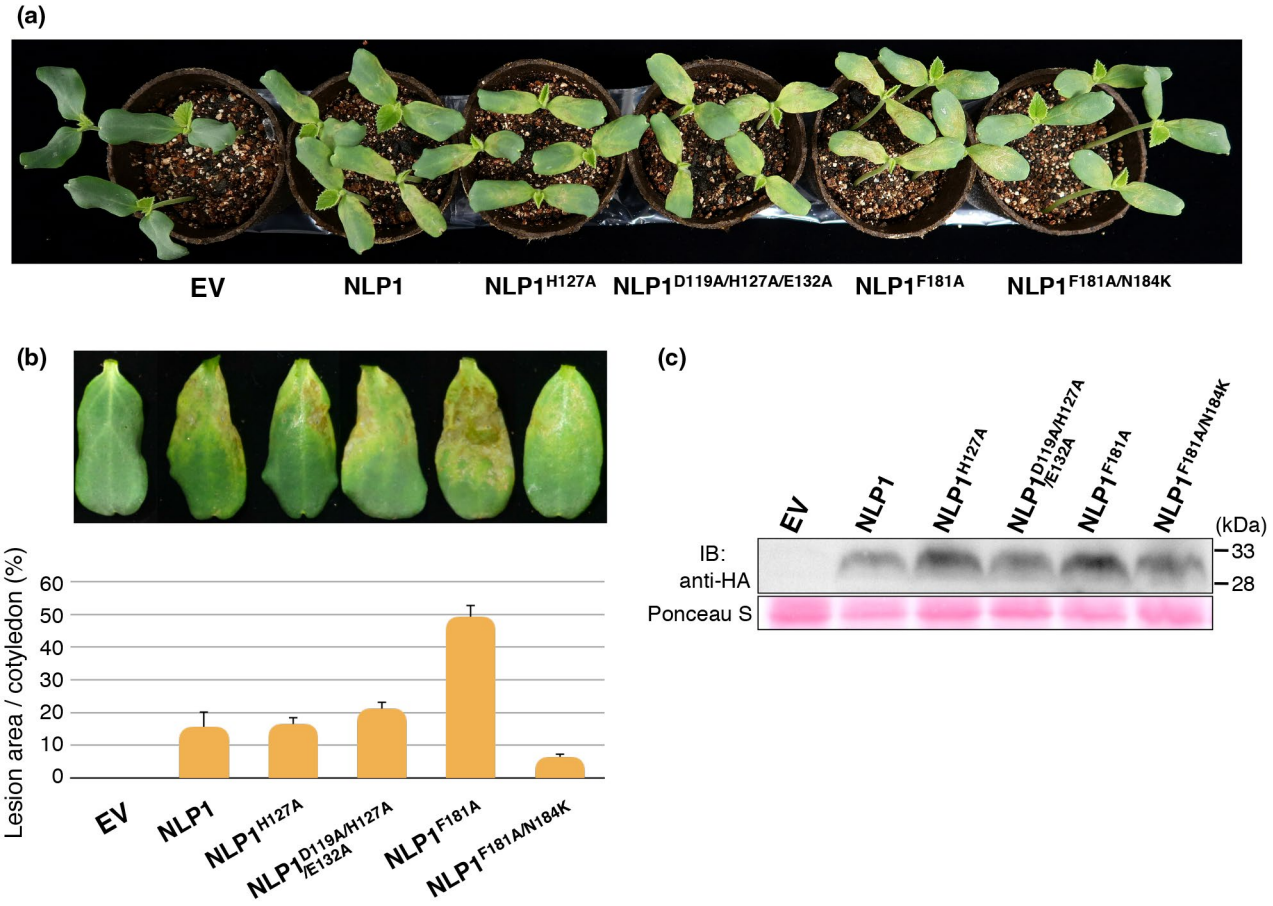

FIGURE 2 Cell death-inducing activity of wild-type and mutated NLP1 proteins expressed in melon. (a) The whole area of melon cotyledons was infiltrated with the *Agrobacteriumtumefaciens* strains shown in Figure 1b. The photograph was taken at 5 days postinfiltration (dpi). (b) Quantitative analysis of necrotic lesion development in infiltrated melon cotyledons from (a). ImageJ was used for measuring the ratios of lesion area in infiltrated cotyledons by adjusting the colour threshold. Means and standard errors were calculated from three independent experiments. (c) Protein extracts at 5 dpi from melon cotyledons expressing the wild-type or mutated NLP1 proteins were analysed by immunoblotting (IB) using anti-HA (3F10; Roche) as the primary antibody and horseradish peroxidase-linked anti-rat IgG (#7077; Cell Signaling Technology) as the secondary antibody

that NLP1^{F181A} lost cell death-inducing activity and that NLP1$^{F181A/N184K}$ markedly reduced the activity in *N. benthamiana* (Figure 1b,c). These observations are consistent with the results of a previous report (Lenarčič et al., 2017). Surprisingly, both NLP1^{F181A} and NLP1$^{F181A/N184K}$ caused yellowish and necrotic symptoms in melon, which contrasted with the case of *N. benthamiana* (Figure 2b,c). These results suggest that the amino acids tested are involved in binding GIPCs and are required for the cell death-inducing activity in *N. benthamiana*, but not in melon. Interestingly, we found that NLP1^{F181A}, but not NLP1$^{F181A/N184K}$, exhibited greater cytotoxic activity than NLP1 in melon (Figure 2b,c).

Based on the finding that all the tested NLP1 mutants caused cell death in melon, we further investigated the cell death-inducing activity of NLP1 lacking its signal peptide (hereafter called NLP1ΔSP) in *N. benthamiana* and melon. We found that the transient expression of NLP1ΔSP failed to cause cell death in *N. benthamiana* (Figure 3a), which is consistent with the previous finding that *Phytophthora sojae* NLP lacking the signal peptide failed to cause cell death in *A. thaliana* (Qutob et al., 2006). Surprisingly, NLP1ΔSP slightly reduced the cell death-inducing activity in melon but still caused cell death clearly (Figure 3b,c), suggesting that the machineries for NLP1-triggered cell death are probably distinct between melon and *N. benthamiana*.

Next, to gain further insights into whether the cell death-inducing activity of *C. orbiculare* effectors differs between *N. benthamiana* and melon, we focused on another conserved effector, necrosis-inducing secreted protein 1 (NIS1), of *C. orbiculare* (Irieda et al., 2019; Yoshino et al., 2012). We previously reported that transient expression of NIS1 caused necrotic lesion formation in *N. benthamiana* (Yoshino et al., 2012). NIS1 is conserved in a broad range of fungi in both Ascomycota and Basidiomycota, where it targets key kinases such as BAK1/SERK3 and BIK1 that are required for plant pattern-triggered immunity (PTI) signalling and suppresses multiple PTI responses, including flg22-triggered generation of reactive oxygen species (ROS) (Irieda et al., 2019). However, the PTI-suppressing activity of NIS1 in cucurbits remains to be elucidated.

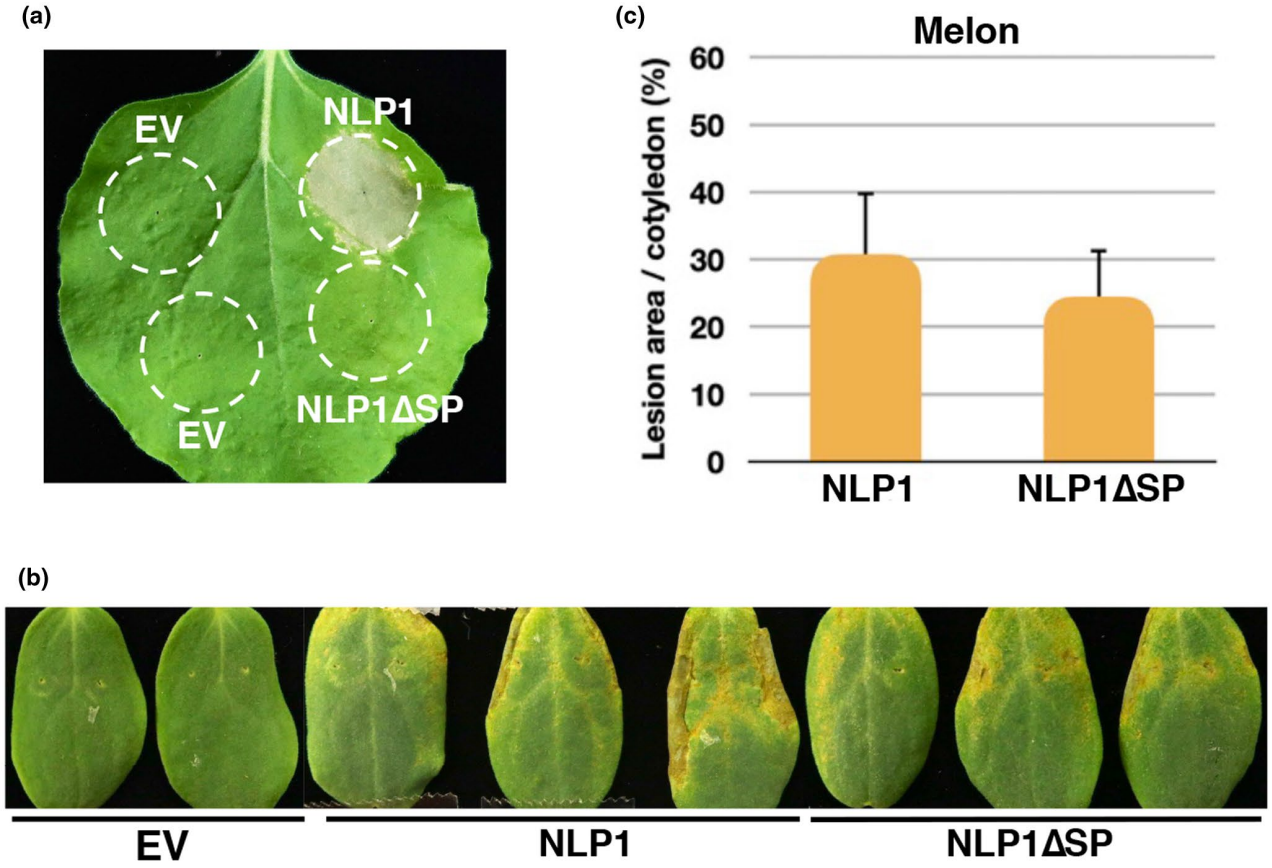

FIGURE 3 NLP1 lacking the signal peptide-induced cell death in melon but not in *Nicotiana benthamiana*. (a) Transient expression of NLP1 without its signal peptide (NLP1ΔSP) failed to cause cell death in *N. benthamiana*. *N. benthamiana* leaves were challenged with *Agrobacterium tumefaciens* GV2260 (gabT) harbouring pEAQ-HT-NLP1:HA and pEAQ-HT-NLP1ΔSP:HA to express NLP1 or NLP1ΔSP. The photograph was taken at 5 days postinfiltration (dpi). (b) Transient expression assay of NLP1 and NLP1ΔSP in melon. The whole area of melon cotyledons was infiltrated with each *A. tumefaciens* strain. The photograph was taken at 5 dpi. (c) Quantitative analysis of necrotic lesion development in infiltrated melon cotyledons from (b)

To investigate this further, we constructed pEAQ-HT-NIS1:HA (Table S1). We first transiently expressed NIS1 in *N. benthamiana* and found that the expression of NIS1 caused necrotic lesions in *N. benthamiana* (Figure 4a,b), a finding that is consistent with our previous report (Yoshino et al., 2012). We then transiently expressed NIS1 in melon cotyledons via agroinfiltration. NIS1 failed to cause cell death in melon cotyledons (Figure 4c). Western blot analysis confirmed the NIS1:HA accumulation in melon cotyledons (Figure 4d). These findings suggest that, in contrast to the observations in *N. benthamiana*, NIS1 has no cell death-inducing activity in melon. We next investigated whether the transient expression of NIS1 suppresses flg22-triggered ROS generation in melon cotyledons. The transient expression of NIS1 markedly reduced flg22-triggered ROS generation (Figure 4e), suggesting that NIS1 can suppress PTI signalling in melon. A mutated NIS1 that lacks the 30 amino acids at the C-terminus (hereafter called NIS1ΔC30) fails to induce cell death in *N. benthamiana* but it suppresses flg22-triggered ROS generation and also associates with BAK1 and BIK1 (Irieda et al., 2019). We investigated the effect of NIS1ΔC30 on flg22-triggered ROS generation in melon and found that NIS1ΔC30 reduced flg22-triggered ROS generation (Figure S3), suggesting that NIS1 suppresses flg22-triggered ROS generation in both melon and *N. benthamiana* in a similar way.

In this study, we established a new efficient system of transient protein expression in melon. Using this new system, we found that two widely conserved fungal effectors, NLP1 and NIS1, from *C. orbiculare* exhibit distinct activities in melon and *N. benthamiana*, both of which this pathogen infects.

In the NLP1 study, we performed mutational analysis on the putative GIPC-binding sites of NLP1 in *N. benthamiana*. Consistent with a previous report (Lenarčič et al., 2017), our results suggest that NLP1 F181 is involved in binding to the cell membrane-exposed GIPCs in *N. benthamiana* (Figure 1b,c). However, the F181A mutation did not cancel the cell death-inducing activity of NLP1 in melon; conversely, the cell death-inducing activity of NLP1^{F181A} was increased in melon (Figure 2a,b).

Cytotoxic NLPs, including NLP1, contain a heptapeptide motif (GHRHDWE) that has been shown to be critical for the cytotoxic activity of NLPs in *N. benthamiana* (Ottmann et al., 2009). The results of our study using NLP1^{H127A} also support this finding. Interestingly, NLP1$^{D119A/H127A/E132A}$ recovered the cytotoxic activity in *N. benthamiana* to some extent (Figure 1b,c). Although it remains

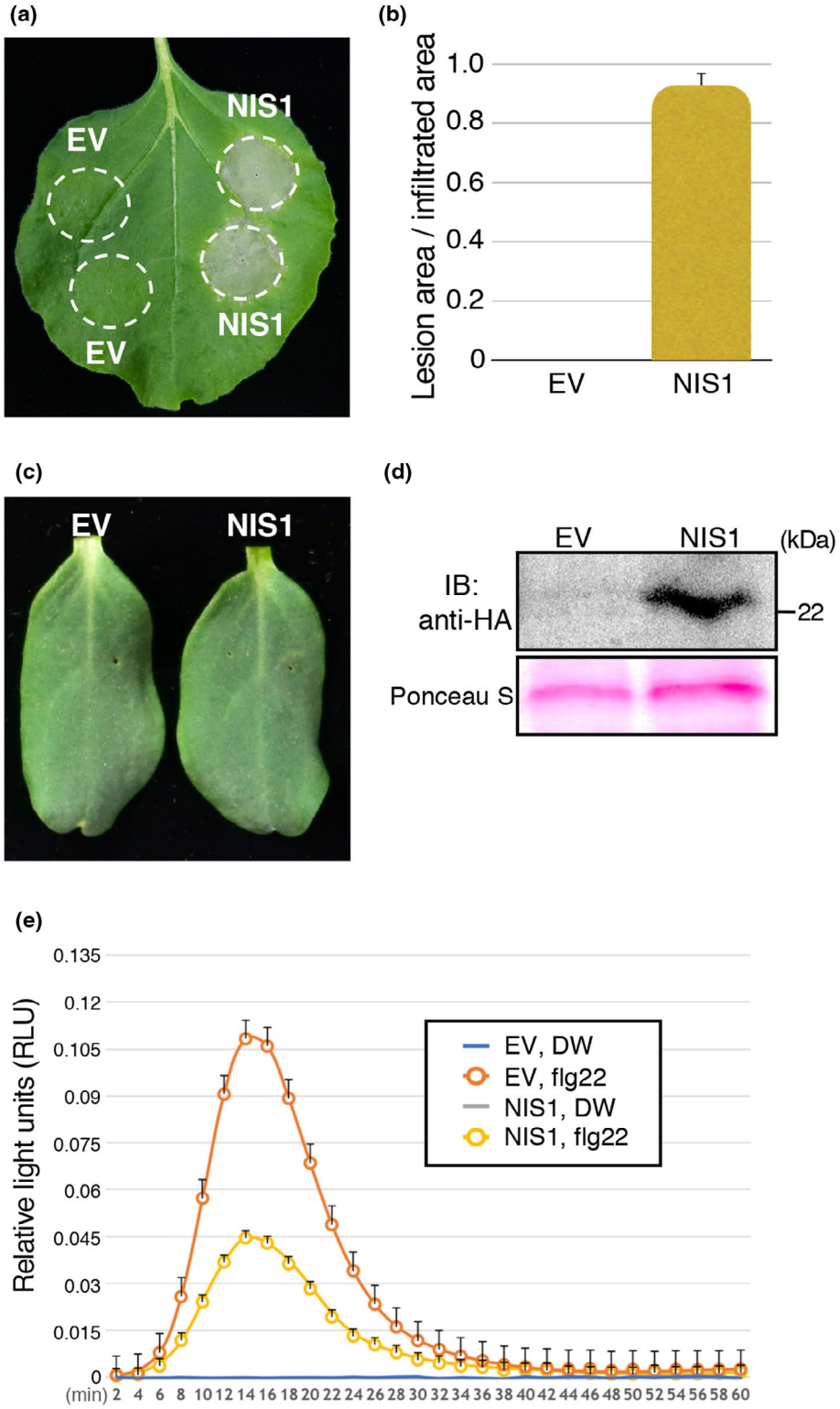

FIGURE 4 NIS1 did not cause cell death but suppressed reactive oxygen species (ROS) generation in melon. (a) *Nicotiana benthamiana* leaves were challenged with *Agrobacteriumtumefaciens* GV2260 (gabT) harbouring pEAQ-HT (empty vector [EV]) or pEAQ-HT-NIS1:HA (NIS1). The photograph was taken at 5 days postinfiltration (dpi). (b) Quantitative analysis of the lesion area per infiltration area in *N. benthamiana*. Means and standard errors were calculated from three independent experiments. (c) NIS1 did not cause development of necrotic lesions in melon. The photograph was taken at 5 dpi. (d) Protein extracts at 5 dpi from melon cotyledons expressing NIS1:HA were analysed by immunoblotting (IB) using an anti-HA antibody. (e) Assay of flg22-triggered ROS generation in melon. Melon cotyledons were infiltrated with *A. tumefaciens* GV2260 (gabT) harbouring pEAQ-HT (empty vector [EV]) or pEAQ-HT-NIS1:HA. At 5 dpi, leaf discs were taken from cotyledons and incubated overnight in distilled water (DW) in the dark, 1 μM flg22 or DW was added, and ROS generation was measured (Irieda et al., 2019). Data are given as relative light units (RLU) and represent the mean ± *SE* ($n = 12$). Similar results were obtained in another independent experiment

to be elucidated how NLP1$^{D119A/H127A/E132A}$ recovered this activity, this finding suggests that D119A and E132A changed the tertiary structure of NLP1^{H127A}, which resulted in the partial recovery of the structure required for the cytotoxic activity in *N. benthamiana*.

Importantly, we found that the point mutations in NLP1 described above did not cancel cell death induction in melon, in contrast to *N. benthamiana*. Why did the tested mutations not abolish the cell death-inducing activity of NLP1 in melon? Surprisingly, we found that NLP1ΔSP induced cell death in melon, whereas NLP1ΔSP completely lacked the cell death-inducing activity in *N. benthamiana* (Figure 3). This finding suggests that apoplastic localization of NLP1 is critical for the cell death induction in *N. benthamiana*, whereas cytoplasmic NLP1 is able to trigger cell death in melon, that is, the machineries for NLP1-triggered cell death are fundamentally distinct between *N. benthamiana* and melon. This idea is further supported by the finding that the point mutations in NLP1 tested in this study did not cancel cell death induction in melon, in contrast to *N. benthamiana*.

We recently reported that *C. orbiculare* transformants constitutively expressing NLP1 failed to develop lesions in multiple cucurbits, including melon (Azmi et al., 2018). However, in contrast to cucurbits, *C. orbiculare* constitutively expressing NLP1 caused lesions in *N. benthamiana* (Azmi et al., 2018), although the transient expression of NLP1 caused severe necrotic lesions in *N. benthamiana* (Figure 1b,c). These findings suggest the possibility that cytoplasmic NLP1 is recognized by unidentified machineries of melon, leading to cell death responses together with activation of immune responses effective to *C. orbiculare*. In the inoculation of *C. orbiculare* transformants constitutively expressing NLP1 on melon, a part of NLP1 secreted by the pathogen might enter into melon cells and strongly activate the host immunity.

In our study, the expression of *C. orbiculare* NIS1 in melon via the new transient expression system showed that NIS1 did not exhibit the ability to induce cell death in melon, in contrast to *N. benthamiana*, even though the pathogen can infect both plants. NIS1 and NIS1ΔC30 suppressed the flg22-elicited ROS burst in both melon (this study) and *N. benthamiana* (Irieda et al., 2019), suggesting that the ability of NIS1 to suppress PTI signalling is conserved in both plants. We previously reported that NIS1-induced cell death depends on both SGT1 and HSP90 (Yoshino et al., 2012), which suggests that NIS1-induced cell death may be triggered via the recognition by an unidentified resistance (R) protein. Therefore, melon may not have the R protein for NIS1 recognition.

Overall, our results show that the cell death-inducing activities and underlying mechanisms of the two *C. orbiculare* effectors differ between *N. benthamiana* and melon, both of which can be infected by this pathogen. For a greater understanding of the effector function of plant pathogens, it will be important to establish new transient expression systems in corresponding host plants in addition to the commonly used *N. benthamiana*.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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