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Insight into Function and Subcellular Localization of a Type III-Secreted Effector in *Pseudomonas syringae* pv. *tomato* DC3000

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Abstract

Pseudomonas syringae pv. *tomato* DC3000 (*Pst* DC3000) is a bacterial pathogen of tomato and of the model plants *Arabidopsis* and *Nicotiana benthamiana* (*N. benthamiana*). Like numerous Gram-negative bacterial pathogens of animals and plants, *Pst* DC3000 exploits the conserved type III secretion system (TTSS) to deliver multiple virulence effectors directly into the host cells. Type III effectors (T3Es) collectively participate in causing disease, by mechanisms that are not well clarity. Elucidating the virulence function of individual effector is fundamental for understanding bacterial infection of plants. Here, we focused on studying one of these effectors, HopAA1-1, and analyzed its potential function and subcellular localization in *N. benthamiana*. Using an Agrobacterium-mediated transient expression system, we found that HopAA1-1 can trigger domain-dependent cell death in *N. benthamiana*. The observation using confocal microscopy showed that the YFP-tagged HopAA1-1 localizes to diverse cellular components containing nucleus, cytoplasm and cell membrane, which was demonstrated through immunoblot analysis of membrane fractionation and nuclear separation. Enforced HopAA1-1 subcellular localization, by tagging with a nuclear localization sequence (NLS) or a nuclear export sequence (NES), shows that HopAA1-1-induced cell death in *N. benthamiana* is suppressed in the nucleus but enhanced in the cytoplasm. Our research is lay a foundation for revealed the molecular pathogenesis of *Pseudomonas syringae* pv. *tomato.*

Keywords

Cell Death, HopAA1-1, *Nicotiana benthamiana*, *Pst* DC3000

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1. Introduction

Pseudomonas syringae pv. *tomato* DC3000 (*Pst* DC3000), which can spawn bacterial speck disease in tomatoes and the model plants *Arabidopsis* and *N. benthamiana*, and has become a model strain for functional genomics and plant-pathogen interaction research [1]. The virulence of *Pst* DC3000 largely depends on the Type III secretion system (T3SS), a complex of proteins encoded by the Hypersensitive Response and Pathogenicity/Hypersensitive Response and Conserved (Hrp/Hrc) genes [2]. The proteins encoded by Hrp/Hrc assemble a device that spans the inner and outer membranes of bacteria, enabling them to directly deliver effectors into the host cytoplasm [2].

Pst DC3000 transmits approximately 30 effectors into plant cells, where collectively suppress plant immune responses and promote bacterial parasitism [3]. The plant immune responses comprise two main branches [4] [5]. The first layer is basal immunity mediated through membrane-localized pattern recognition receptors (PRRs), known as pattern-triggered immunity (PTI). When PTI is overcome by pathogens, the second layer of immunity is then activated. The intracellular nucleotide-binding leucine-rich repeat receptors (NLRs) initiate the second layer of immunity through directly or indirectly to recognize the released effectors from pathogens, and this immunity is termed effector-triggered immunity (ETI). ETI frequently results in local programmed cell death and induces hypersensitivity response (HR) [6].

By analyzing the genome sequences of 494 strains of *Pseudomonas syringae* (*P. syringae*), the complexity, diversity, and abundance of T3SE were uncovered [7]. Interestingly, although each strain of *P. syringae* has a unique number and library of effectors, four core effectors were identified in over 95% of the strains: AvrE, HopB1, HopM1, and HopAA1, highlighting their importance in basic interactions or pathogenesis with plants [1]. The function of the remaining effectors in each strain is difficult to assess for the following reasons: 1) a given effector can have several targets, 2) multiple effectors are redundant, having the same function, or targeting the same plant component, and 3) effectors can interact with other effectors in multiple combinations. The generation of *Pst* DC3000 poly-mutants with sequential deletion of its well-expressed 28 effectors and further combinatorial reassembly of effectors has significantly contributed to untangle the complexity of *Pst* DC3000 by evaluating their growth in *N. benthamiana*. *Pst* DC3000 effectors are functionally redundant because mutations in individual effectors do not sensibly affect pathogenicity, but all effectors are necessary for complete virulence in tomato and *Arabidopsis* [8]. Previous studies have shown that only eight effectors, including HopAA1-1, constitute the minimal functional set of effectors required for the virulence of *Pst* DC3000 in *N. benthamiana* [9]. Among those effectors, HopAA1-1 colocalized with porin to yeast mitochondria and was shown to mediate cell death in yeast and plants in a domain-dependent manner [10]. However, further research is needed on the localization and function of HopAA1-1 in plants.

Here, we focused on studying HopAA1-1 and analyzing its potential function and subcellular localization in plants. Through transient expression in *N. benthamiana*, we observed that HopAA1-1 can trigger domain-dependent cell death. The observation using confocal microscopy showed that the YFP-tagged HopAA1-1 localizes to a variety of cellular compartments, including nucleus, cytoplasm and cell membrane, which was demonstrated through immunoblot analysis of membrane fractionation and nuclear separation. Enforced HopAA1-1 subcellular localization, by tagging with a nuclear localization sequence (NLS) or a nuclear export sequence (NES), shows that HopAA1-1 activity in cell death signaling is suppressed in the nucleus but enhanced in the cytoplasm. Our research lays a foundation for revealing the molecular pathogenesis of *Pseudomonas syringae* pv. *tomato.*

2. Materials and Methods

2.1. Experimental Materials and Growth Conditions

Pst DC3000 was grown on King's B (KB) medium containing rifampicin (25 μg/ml). *N. benthamiana* plants were grown in a greenhouse at 24˚C, 12 h light/12 h dark, and 70% relative humidity. The 5-week-old *N. benthamiana* plants were used for subcellular localization and transient expression analyses.

2.2. Plasmid Constructs

The plasmid vectors were constructed as mentioned in previous studies [11]. In brief, the amplified DNA fragments of HopAA1-1 and the truncations were cloned into the entry vector pENTR/D-TOPO (Thermo Fisher Scientific), and then transferred to the expression vector pEarleygate101 for YFP (Yellow Fluorescent Protein)-HA labeled protein expression, or the modified pEarleygate101 for YFP-HA-NES, YFP-HA-nes, YFP-HA-NLS and YFP-HA-nls tagged protein expression.

2.3. Transient Expression in *N. benthamiana*

Transient expression in *N. benthamiana* as described previously [12]. Briefly, expression vectors were transformed into *Agrobacterium* GV3101 and cultured for 10 - 12 hours. The cultured *Agrobacterium* were centrifuged and re-suspended in MES buffer (10 mM MES pH 5.6, 10 mM $MgCl_2$, and 150 μM acetosyringone) for 1 hour (h). The diluted suspensions were injected into the leaves of 5-week-old *N. benthamiana* plants. After 48 h of infiltration, the leaves of the plants were used for protein extraction.

2.4. Western Blot

N. benthamiana transiently expressing proteins of interest were collected in liquid nitrogen. Tissue was ground in liquid nitrogen and homogenized with protein extraction buffer (20 mM Tris-HCl pH 8.0, 5 mM ethylene diamine tetraacetic

acid (EDTA), 1% SDS, and 10 mM DL-dithiothreitol (DTT)). Lysate was boiled at 98˚C with 1× protein loading buffer for 10 min. The total protein extract was cleared by centrifuge at 15,000 g for 10 min. Then, the supernatant was separated by 10% SDS–PAGE gels and detected with corresponding antibodies.

2.5. Nuclear-Cytoplasmic Protein Fractionation

Nuclear and cytoplasmic proteins were extracted according to a previously published method [13]. Briefly, 1 g of plant leaves was ground in liquid nitrogen and transferred into 4 mL of buffer A. The extracted proteins were infiltrated through two layers of Mira-cloth and centrifuged at 1500 g at 4˚C for 20 minutes (min) to separate the crude cytoplasmic and nuclear fractions. Subsequently, the supernatant was centrifuged at 16,000 g at 4˚C for 15 min to obtain the cytoplasmic protein. The precipitate was washed with buffer B and buffer C, followed by centrifugation at 16,000 g at 4˚C for 20 min. The final precipitate was re-suspended in 200 μL of nuclear lysis buffer. The primary antibodies used in this study: Anti-HA (Roche, #11867423001) and Anti-Histone H3 (Abcam, #ab1791).

2.6. Membrane Fractionation Assays

In brief, sucrose buffer [20 mM Tris (pH 8.0), 0.33 M sucrose, 1 mM EDTA, 5 mM DTT, and 1× Sigma plant protease inhibitor cocktail] was added to the homogenized tissue at a ratio of 5 μL per mg (FW) tissue. The extract was centrifuged at 5000 g for 10 min at 4˚C; then, the supernatant was transferred to a new tube and designated as total protein (T). Cytoplasmic fraction (C) was prepared by harvesting the supernatant after spinning the total protein fraction at 20,000 g for 1 h at 4˚C. The total membrane fraction was prepared from the resulting pellet by re-suspending in 200 μL of buffer B (Minute^{TM} PM protein isolation kit, Invent Biotechnologies). After centrifugation at 7800 g for 15 min at 4˚C, the supernatant was transferred to 2 mL Eppendorf tube and mixed with 1.6 mL cold phosphate balanced solution (PBS) buffer mixed by vibrate and spun at 16,000 g for 1 h at 4˚C to pellet the plasma membrane (PM) fraction. The resulting fraction was labeled as the PM-enriched/microsomal fraction. The primary antibodies used in this study: Anti-HA (Roche, #11867423001) and anti-H^+-ATPase (Agrisera, #AS07260).

2.7. Confocal Microscopy

Subcellular localization was observed following a previously mentioned method [14]. In a nutshell, the images were observed on the abaxial sides of leaves at 28 h post-target protein expression with a confocal microscope (Leica SP8). YFP fluorescence was excited at 514 nm.

2.8. Trypan Blue Staining

The transiently expressed leaves of *N. benthamiana* were placed into blue cap tubes containing 30 mL trypan blue stain solution. The leaves were boiled for 5

min and stained at room temperature for 30 min. The stained leaves were washed with chloral hydrate solution several times to get clear background.

3. Results

3.1. HopAA1-1 Expression Causes Virulence-Related Cell Death in *N. benthamiana*

Plant cell death is related to the non-toxic activity of *P. syringae* effectors in resistant plants and the formation of lesions in susceptible plants. Therefore, identifying effectors that can cause non-host or host plant cell death is a useful first step in cataloging the potential functions of effector libraries. We generated several effector genes from *Pst* DC3000, and transiently expressed them in the leaves of *N. benthamiana* to determine these effectors that might play a role in virulence-related cell death. We observed that the HopAA1-1 was able to trigger cell death in *N. benthamiana* (Table 1 and Figure 1(a)).

Table 1. Assay for ability of the several effectors from *Pst* DC3000 to elicit cell death in *N. benthamiana*.

Pst DC3000 effector	HR-like cell death phenotypes
AvrE1	+
HopA1	−
HopF2	−
HopH1	−
HopR1	−
HopX1	+
HopAA1-1	+
HopAM1-1	−

Ulteriorly, we want to ascertain the fragments of HopAA1-1 needful for cell death in *N. benthamiana*. Successive truncations were produced from both ends of the gene (Figure 1(b)) and similar levels of expression was detected for each by immunoblotting assay (data not shown). Only HopAA1-1 derivatives with relatively small deletions, 40 amino acids from the N-terminus and 50 amino acids from the C-terminus of the protein, maintained the ability to provoke cell death in *N. benthamiana* (Figure 1(c)). To sum up, HopAA1-1 can strike domain-dependent cell death in *N. benthamiana.*

3.2. HopAA1-1 Localizes to Multiple Intracellular Sites in *N. benthamiana*

Subcellular localization is closely related to protein function [15]. We examined the subcellular positioning of HopAA1-1 with a confocal microscope based on the C-terminal YFP-HA tag, and discovered that HopAA1-1-YFP-HA is localized

multiple compartments including nucleus, cell membrane, and cytoplasm (Figure 2(a)).

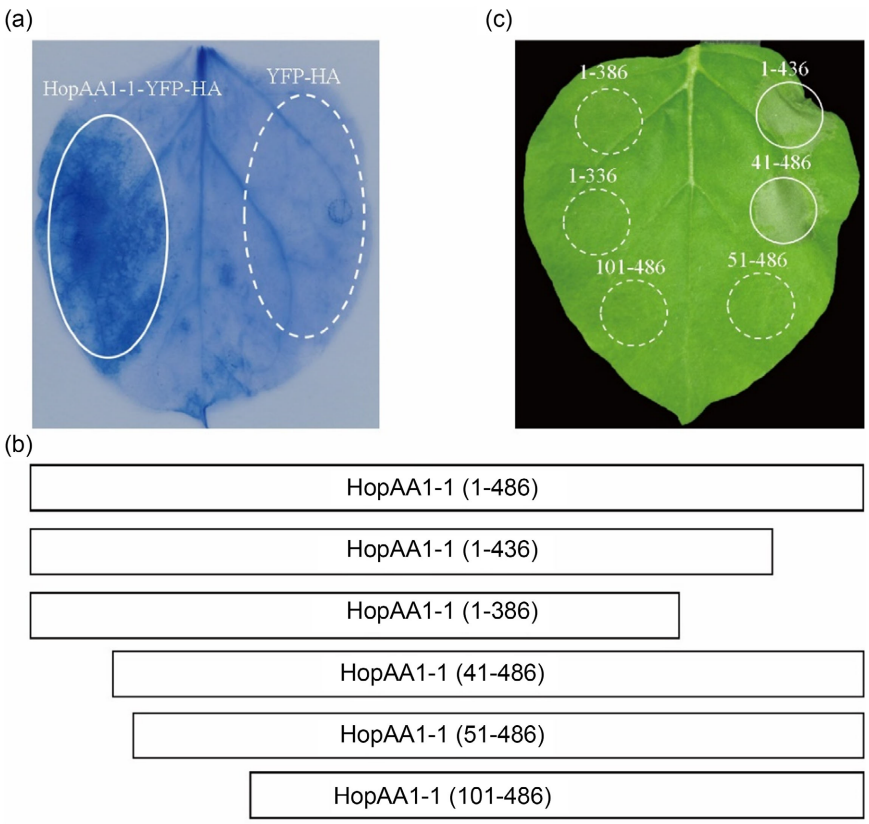

Figure 1. Domain-dependent cell death triggered by HopAA1-1 in *N. benthamiana*. (a) Transiently expressed HopAA1-1 sparks cell death. *35S:: HopAA1-1-YFP-HA* was transiently expressed in *N. benthamiana* by Agrobacterium infiltration (OD_{600} = 0.8). *35S:: YFP-HA* was served as a negative control. Visible cell death was corroborated by trypan blue staining. Picture was photographed at 48 h post infiltration (hpi). The experiment was repeated at least three times with similar results. (b) The indicated HopAA1-1 derivatives were cloned into pEarleyGate101-YFP-HA and were transiently expressed in *N. benthamiana*. (c) Analysis of cell death-inducing activity of HopAA1-1 fragments. The experiment was repeated at least three times with similar results.

To verify the fluorescence data, we conducted membrane fractionation and nuclear separation experiments. We employed H^+-ATPase, Histone H3 and Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) work as markers of membrane, nuclear, and soluble components, respectively. We first separated the total protein (T) extraction into nuclear depleted (ND) and nuclear enriched (NE) fractions. The biochemical results showed that quite amount of HopAA1-1 was distributed in the nucleus (Figure 2(b)). The ND fraction was further separated into the pellet membrane fraction and the supernatant cytosol fraction, and HopAA1-1 was predominantly detected in the membrane fraction (Figure 2(c)). To summarize, HopAA1-1 localizes to multiple intracellular sites, including the cytoplasm, cell membrane and nucleus in *N. benthamiana*.

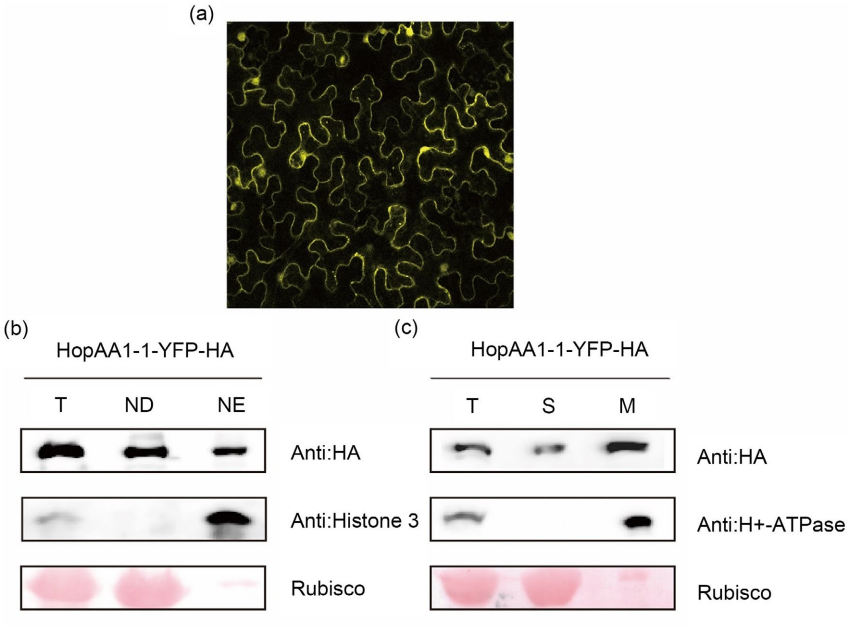

Figure 2. HopAA1-1 localizes to multiple intracellular sites, including the cytoplasm, cell membrane and nucleus. (a) Observation the subcellular localization of HopAA1-1 with fluorescence microscope. Photographs were derived at 28 h post infiltration (hpi). (b) Nuclear localization of HopAA1-1 was verified by nuclear-cytoplasmic protein fractionation. Total protein extractions (T) were separated into nuclear deleted (ND) and nuclear enrich (NE) fractions with centrifugation. The distribution of HopAA1-1 was detected with anti-HA antibody. Histone 3 and Rubisco were used as the markers of NE and ND fraction, respectively. (c) The membrane and cytosol distribution of HopAA1-1. Total protein extractions (T) were separated into membrane (M) and cytosol (S) two fractions. H^+-ATPase and Rubisco were employed as membrane marker and cytosol marker, respectively.

3.3. Distinct Nuclear and Cytoplasmic HopAA1-1 Activities in Cell Death Signaling

We have demonstrated that HopAA1-1 localizes to multiple intracellular sites. Next, we are interested in the relationship between the subcellular partitioning of HopAA1-1 and its cell death-inducing activity. We created two pairs of HopAA1-1 fusions, HopAA1-1-YFP-HA-NES vs. HopAA1-1-YFP-HA-nes (NES is a nuclear export sequence, "nes" is a mutated nonfunctional NES), and HopAA1-1-YFP-HA-NLS vs. HopAA1-1-YFP-HA-nls (NLS is a nuclear localization sequence, while "nls" is a mutated NLS, serving as a negative control) [16]. Upon expression of these HopAA1-1 versions in *N. benthamiana* leaf, laser confocal microscope observation revealed that the NES tagged HopAA1-1 was mainly distributed outside the nucleus. Nevertheless, the NLS labeled HopAA1-1 was almost only detected in the nucleus. The fluorescence signal of the HopAA1-1-YFP-HA-nes and HopAA1-1-YFP-HA-nls variants clearly partitioned to both nucleus and cytoplasm, similar to that of HopAA1-1-YFP-HA (**Figure 3(a)**). Remarkably, the expression of HopAA1-1-YFP-HA-NES triggered a cell death response that was more severe than the one initiated by HopAA1-1-YFP-HA-nes, whereas the cell death mediated by HopAA1-1-YFP-HA-NLS was obviously weaker than that of

the control group HopAA1-1-YFP-HA-nls (Figure 3(b)). In a word, the activity of HopAA1-1 in cell death signaling is inhibited in the nucleus but strengthened in the cytoplasm.

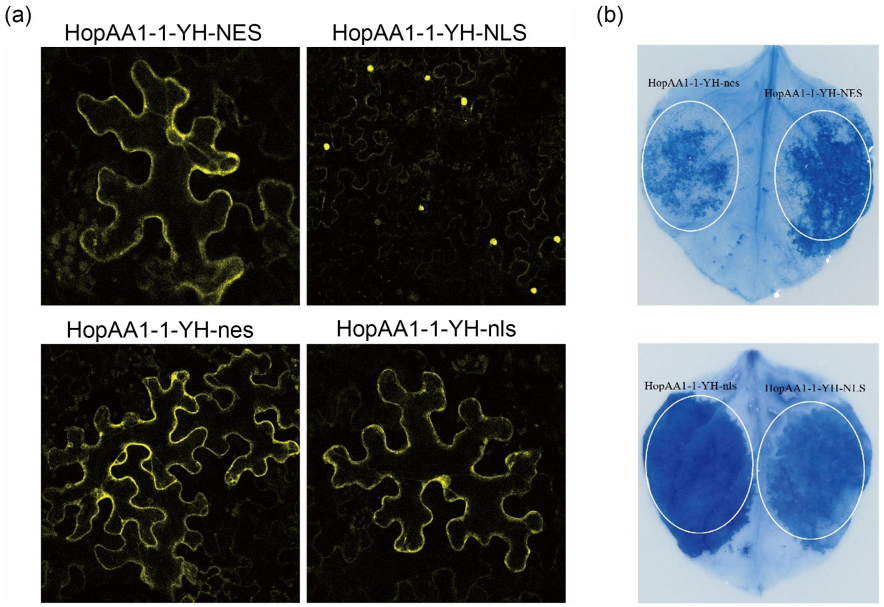

Figure 3. Distinct nuclear and cytoplasmic HopAA1-1 activities in cell death signaling. (a) Confocal images of *N. benthamiana* leaf expressing HopAA1-1 fusion proteins. The confocal images were taken at 28 hpi. NES: nuclear export sequence; nls: mutated nonfunctional NES; NLS: nuclear localization signal; nls: mutated nuclear localization signal. (b) Analysis of cell death triggering activity of HopAA1-1 fusion proteins. Indicated HopAA1-1 fusion proteins were expressed in *N. benthamiana* leaves by Agro-infiltration, and cell-death triggered by each fusion protein was scored by trypan blue staining at 40 hpi.

4. Discussion

The parasitic success of *Pseudomonas syringae* is believed to hinge on the ability of type III effectors to inhibit PTI response, meanwhile escaping (or suppressing) detection by ETI reaction [5]. The molecular functions of AvrPto and HopM1 offer some cases of effectors interfering with at least two prominence processes in PTI: PAMP perception and cell wall defense-associated vesicle trafficking, respectively [17]-[19]. Moreover, AvrPtoB provides an instance of an effector with several modules that might function to inhibit PTI and to both provoke and suppress ETI [20]. We have demonstrated that HopAA1-1 can initiate domain-dependent cell death in *N. benthamiana* (Figure 1). Combined with the previous discovery of transiently expressed HopAA1-1 acts inside *Saccharomyces cerevisiae* to spawn cell death [10]. Although PAMP perception concerns particular PRRs kinases and signaling components that are unlikely to be found in yeast, defense-related vesicle trafficking may be ubiquitous in eukaryotes. Therefore, the widespread toxicity of the transiently expressed HopAA1-1 to yeast and plant cells would be consistent with the main role of this effector in disrupting basic cellular processes such as vesicle transport. Noteworthy, the *Pst* DC3000 HopAA1-1 expressed in *Arabidopsis*

protoplasts was capable of restraining flagellin-induced accumulation of NON-HOST1 (NHO1) transcripts, a marker for PTI-related gene expression [21]. The findings declare that HopAA1-1 can interdict PAMP perception, although it is probable that secondary effects of our proposed disruption of a fundamental cellular process like vesicular transport by HopAA1-1 contributed to this found.

HopAA1-1 localizes to multiple intracellular sites in *N. benthamiana* (**Figure 2**). These subcellular regions may represent the various biological functions performed by the HopAA1-1. Correspondingly, NLRs also exhibit diverse positioning. RPM1 (Resistant to *Pseudomonas syringae* pv. *maculicola* 1) and RPS5 (Resistant to *Pseudomonas syringae* 5) are localized on the plasma membrane [22] [23]. L6 and M proteins are localized on the Golgi and tonoplast, respectively [24]. RGA4 (R-gene analog 4) and RGA5 are mainly localized in the cytosol [25]. SNC1 (Suppressor of npr1-1, constitutive 1), Rx (Resistance to *Potato virus X* (PVX)), MLA10 (Mildew locus a 10) and RppM (Resistance to *Puccinia polysora* Maize) show a nucleo-cytoplasmic localization [16] [26]-[28]. Our study shows that HopAA1-1 activity in cell death signaling is suppressed in the nucleus but enhanced in the cytoplasm (**Figure 3**). An unresolved issue is whether the cytoplasmic HopAA1-1 pool alone is enough to arise cell death.

5. Conclusion

Collectively, this study presents novel insight into function and subcellular localization of HopAA1-1 from Pst *DC3000*i within *N. benthamiana*, a model plant system. Using an Agrobacterium-mediated transient expression system, we found that HopAA1-1 can trigger domain-dependent cell death in *N. benthamiana*. The observation using confocal microscopy showed that the YFP-tagged HopAA1-1 localizes to diverse cellular components containing nucleus, cytoplasm and cell membrane, which was demonstrated through immunoblot analysis of membrane fractionation and nuclear separation. Enforced HopAA1-1 subcellular localization, by tagging with a nuclear localization sequence (NLS) or a nuclear export sequence (NES), shows that HopAA1-1-induced cell death in *N. benthamiana* is suppressed in the nucleus but enhanced in the cytoplasm. These findings could pave the way for a deeper understanding of the molecular pathogenesis of *Pseudomonas syringae* pv. *tomato.*

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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