Dysfunction of Arabidopsis MACPF domain protein activates programmed cell death via tryptophan metabolism in MAMP-triggered immunity

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Received 24 February 2016; revised 30 September 2016; accepted 3 October 2016; published online 7 January 2017.

SUMMARY

Plant immune responses triggered upon recognition of microbe-associated molecular patterns (MAMPs) typically restrict pathogen growth without a host cell death response. We isolated two Arabidopsis mutants, derived from accession Col-0, that activated cell death upon inoculation with nonadapted fungal pathogens. Notably, the mutants triggered cell death also when treated with bacterial MAMPs such as flg22. Positional cloning identified \textit{NSL1} (\textit{Necrotic Spotted Lesion 1}) as a responsible gene for the phenotype of the two mutants, whereas \textit{nsl1} mutations of the accession No-0 resulted in necrotic lesion formation without pathogen inoculation. \textit{NSL1} encodes a protein of unknown function containing a putative membrane-attack complex/perforin (MACPF) domain. The application of flg22 increased salicylic acid (SA) accumulation in the \textit{nsl1} plants derived from Col-0, while depletion of isochorismate synthase 1 repressed flg22-inducible lesion formation, indicating that elevated SA is needed for the cell death response. \textit{nsl1} plants of Col-0 responded to flg22 treatment with an RBOHD-dependent oxidative burst, but this response was dispensable for the \textit{nsl1}-dependent cell death. Surprisingly, loss-of-function mutations in \textit{PEN2}, involved in the metabolism of tryptophan (Trp)-derived indole glucosinolates, suppressed the flg22-induced and \textit{nsl1}-dependent cell death. Moreover, the increased accumulation of SA in the \textit{nsl1} plants was abrogated by blocking Trp-derived secondary metabolite biosynthesis, whereas the \textit{nsl1}-dependent hyperaccumulation of PEN2-dependent compounds was unaffected when the SA biosynthesis pathway was blocked. Collectively, these findings suggest that MAMP-triggered immunity activates a genetically programmed cell death in the absence of the functional MACPF domain protein NSL1 via Trp-derived secondary metabolite-mediated activation of the SA pathway.

Keywords: MACPF domain, MAMPs, cell death, Tryptophan-derived metabolism, salicylic acid signaling, secondary metabolites, \textit{Colletotrichum orbiculare}.

INTRODUCTION

Effective innate immunity in higher plants often depends on the activation of two interconnected immune responses that limit or terminate pathogen growth. Activation of immune responses mediated by the recognition of microbe-associated molecular patterns (MAMPs) on the plant cell surface is referred to as MAMP-triggered immunity (MTI) and limits pathogen proliferation. Several membrane-resident receptor-like kinases, including FLS2, EFR, and CERK1, have been characterized as MAMP receptors for the extracellular detection of evolutionarily broadly conserved microbial epitopes present in bacterial flagellin, the translation elongation factor EF-Tu or the fungal cell wall component chitin, respectively (Boller and Felix, 2009; Schwessinger and Ronald, 2012).
MTI is generally sufficient to terminate infection attempts of nonadapted pathogens. However, host-adapted pathogens can suppress MTI by the delivery of a repertoire of virulence factors, called effectors, which act either extracellularly or inside host cells. As counter-defense mechanism against this effector-mediated infection strategy, intracellular nucleotide-binding domain and leucine-rich repeat containing receptors (NLRs) re-activate immune responses upon the detection of often strain-specific pathogen effectors. NLRs detect the presence of cognate effectors inside host cells either directly or indirectly through effector-mediated modifications of host targets (Dodds and Rathjen, 2010; Maekawa et al., 2011). The detection of pathogen-derived effectors by NLR immune receptors initiates a rapid, and local immune response, known as effector-triggered immunity (ETI) (Tsuda and Katagiri, 2010). In many cases ETI is associated with a form of programmed cell death (PCD), which is called the hypersensitive response (Heath, 2000a). By contrast, MTI-mediated defense responses are transient and usually not associated with a PCD. Interestingly, although the output of MTI is normally different from that of ETI, both branches overlap significantly with each other in terms of host gene expression and defensive traits (Navarro et al., 2004; Tsuda and Katagiri, 2010).

We aim to identify molecular components and pathways underlying durable resistance of *Arabidopsis thaliana* toward nonadapted pathogens, called nonhost resistance (Heath, 2000b; Schulze-Lefert and Panstruga, 2011). We are particularly interested in *Colletotrichum* fungal species that exhibit a hemibiotrophic infection strategy (Perfect et al., 1999; Shimada et al., 2006). In hemibiotrophic interactions the fungus initially invades and retrieves nutrients from living host cells and switches to a necrotrophic colonization mode at later infection stages by killing host cells for nutrient retrieval. To identify factors involved in nonhost resistance of *Arabidopsis* against *C. orbiculare* that infects *Cucurbitaceae* plants in nature (Gan et al., 2013; Kubo and Takano, 2013), we screened for *Arabidopsis* mutants that develop leaf lesions upon *C. orbiculare* inoculation. The mutant survey identified two allelic mutants in accession Col-0 that we named *lic1* (*lesion induced by Colletotrichum*). Further analysis revealed that mutations in *At1g28380* were responsible for the *lic1* phenotype. Two independent mutations in the same gene have been previously reported to be responsible for the *nsl1* (*necrotic spotted lesion*) phenotype in the Arabidopsis accession No-0 (Noutoshi et al., 2006). Both *nsl1* mutants (nsl1-1 and nsl1-2) of the accession No-0 show severe growth retardation and spontaneous necrotic leaf lesions, which is not the case for both *lic1*-1 and *lic1*-2 mutants of the accession Col-0, renamed *nsl1*-3 and *nsl1*-4, respectively.

*Nsl1* encodes a protein containing a putative membrane-attack complex/perforin (MACPF) domain. It is known that several mammalian MACPF domain proteins play important roles in immunity by forming pores in the cell membranes of pathogenic parasites, such as Gram-negative bacteria, or in the membranes of virus-infected host cells, thereby causing these cells to burst (Rosado et al., 2008; McCormack et al., 2013). Structural determination of the MACPF domain revealed that MACPF is structurally homologous to the pore-forming toxins of Gram-positive bacteria, called cholesterol-dependent cytolysins (Rosado et al., 2007). However, there is little information about the molecular function of MACPF domain proteins, including Nsl1, in higher plants.

Here we generated stable transgenic Arabidopsis lines expressing functional green fluorescent protein (GFP)-Nsl1 fusion protein and revealed that Nsl1 localizes to the plasma membrane of *Arabidopsis* leaf cells. We found that the application of peptide bacterial MAMPs such as flg22 (a 22-amino acid peptide corresponding to a conserved epitope of bacterial flagellin) or elf18 (elongation factor Tu N-acetylated peptide, comprising the first 18 amino acids of the N-terminus) also induced leaf lesions in the *nsl1*-3 and *nsl1*-4 mutants (Col-0 background). These findings indicated that, in wild-type Arabidopsis, MAMP-induced cell death is repressed by the plasma membrane-associated MACPF protein. We then dissected the genetic circuitry underlying the MAMP-induced cell death response in the *nsl1* mutants and found that loss-of-function mutations in penetration 2 (*Pen2*) largely abolish the MAMP-induced and *nsl1*-dependent cell death. *Pen2* is known to be essential for the pathogen-inducible metabolism of Trp-derived indole glucosinolates (IGs) and nonhost resistance of *Arabidopsis* against multiple nonadapted fungal pathogens (Lipka et al., 2005; Hiruma et al., 2010). Our findings reveal an unexpected link between pathogen-induced antimicrobial metabolism and MAMP-induced cell death. Further analyses revealed that antimicrobial metabolites activate salicylic acid (SA) signaling to trigger cell death during MTI in the absence of the functional MACPF domain protein.

**RESULTS**

**Mutations in Arabidopsis NSL1 lead to the development of necrotic leaf lesions upon *C. orbiculare* inoculation**

To identify genetic components contributing to nonhost resistance in *A. thaliana* we screened an *Arabidopsis* mutant population for plants that formed leaf lesions upon inoculation with nonadapted *C. orbiculare* (*Co*), which in nature colonizes cucumber (Kubo and Takano, 2013). Among about 6000 ethyl methanesulfonate mutagenized *M₂* Col-0 plants we identified two independent Arabidopsis mutants, designated L1 and L2, that formed necrotic leaf lesions accompanied by whole leaf yellowing upon inoculation with *Co* spores (Figure 1(a)). F₁ progeny obtained...
from crosses between L1 and L2 mutants exhibited the same necrotic leaf phenotype upon Co challenge, suggesting that both mutants might carry allelic gene defects, designated lic1 (lesion induced by Colletotrichum 1). Positional cloning using F2 populations obtained from crosses between L2 (lic1-2) and the accession Ler mapped the lic1 allele between SGCSNP 9883 (9880523 bp) and SGCSNP9897 (10007758 bp) on chromosome 1.

DNA polymorphisms between Col-0 and the two lic1 mutants of this region were investigated using the TILLING (targeting induced local lesions in genomes) method (McCallum et al., 2000). The L1 (lic1-1) and L2 (lic1-2) polymorphisms were found in the same gene At1g28380 previously named Necrotic Spotted Lesions1 (NSL1) (Noutoshi et al., 2006) (Figures 1(b) and S1). Sequencing of the entire region of NSL1 revealed that L1 has a mutation at nucleotide position 420 that changes Gly to Arg at amino acid residue 109, whereas L2 has a mutation at nucleotide position 538 that changes Gln to stop at amino acid residue 538. We concluded that these NSL1 mutations are responsible for the lic1 phenotype. Previously, two independent transposon insertions into NSL1 (nsl1-1 and nsl1-2) in the Arabidopsis accession No-0 resulted in dwarfism and leaf lesions in the absence of microbial pathogens (Noutoshi et al., 2006), which is distinct from the phenotype of L1 (lic1-1) and L2 (lic1-2) exhibiting no lesion phenotype in the absence of pathogens (Figure 1(a)). We hereafter call lic1-1 and lic1-2 as nsl1-3 and nsl1-4, respectively. NSL1 encodes a protein of unknown function that contains a putative MACPF domain (Rosado et al., 2008; McCormack et al., 2013). The nsl1-3 mutation affects in the NSL1 gene produce an amino acid adjacent to the N-terminal end of a deduced MACPF domain whereas the nsl1-4 mutation predicts a truncation at a distance from this domain close to the NSL1 C-terminus (Figures 1(b) and S1).

Colletotrichum gloeosporioides (Cg; strain S9205) infects mulberry but cannot infect Arabidopsis because Cg entry attempts of fungal germlings into leaf epidermal cells are terminated by pathogen-inducible extracellular defense responses (Shimada et al., 2006; Hiruma et al., 2010). We previously reported that nonadapted Cg grows invasively in Arabidopsis pen2 plants that are defective in pre-invasive resistance responses, and that subsequent invasive growth is accompanied by leaf lesion formation (Lipka et al., 2005; Hiruma et al., 2010). We found that Cg also induced leaf lesions on the nsl1-3 mutant. In addition, a leaf yellowing was seen outside the Cg-inoculated area of nsl1-3 plants, whereas any macroscopically detectable infection symptoms were confined to the droplet inoculated leaf area in the pen2 mutant (Figure 1(c)).

We determined fungal entry rates of Cg germlings into nsl1-3 leaf epidermal cells and found these were similar to entry rates on wild-type (WT) plants (close to 0%), whereas...
fungal entry on pen2 plants was about 20% (Figure 1(d)). These results indicate that nonhost resistance of the ns1-1 plants in entry control against Cg is fully retained although Cg inoculation induced necrotic lesion development. This uncoupling of lesion development from Cg invasion pointed to the possibility that cell death was improperly activated in the ns1-1 mutants coincident with pre-invasive pathogenesis of nonadapted Co and Cg. As mentioned, the ns1-1 and ns1-2 mutants of the accession No-0 developed dwarfism and leaf lesions in the absence of microbial pathogens (Noutoshi et al., 2006). Although the ns1-3 and -4 mutants of Col-0 were neither dwarfed nor developed leaf lesions in pathogen-free environments, our findings and those reported earlier suggest that NSL1 (At1g28380) has a role in cell death repression in the absence of pathogens as well as during pathogen-triggered plant defense.

MAMP-induced cell death in the ns1-1 mutants

The finding that ns1-1-dependent leaf lesion formation in Col-0 is uncoupled from pathogen invasion raised the possibility that extracellular pathogen recognition is sufficient for the activation of ns1-1-dependent cell death. The presence of a number of well-characterized MAMPs, including the bacterium-derived flg22 and elf18 epitopes, is recognized by corresponding membrane-resident pattern recognition receptors (PRRs) on the host cell surface, which initiates MTI (Boller and Felix, 2009; Schwessinger and Ronald, 2012). We investigated whether MAMP treatment can induce the lesion phenotype in the ns1-1-3 and ns1-1-4 mutants by administering droplets containing a bacterium-derived MAMP on the leaf surface of the tested plant lines. As a result, treatment with flg22 at 10 μM resulted in clear necrotic lesions in mature leaves and cotyledons on both ns1-1 mutant lines but not WT plants (Figure 2(a)). Lesions also developed on cotyledons of the ns1-1 mutant seedlings upon treatment with the same MAMP (Figure 2(b)). Infiltration of flg22 at 1 μM also induced lesion development in ns1-1-3 but not in WT (Figure S2(b)). Droplet application of 10 μM elf18 triggered lesion formation in the mutants but not in WT plants (Figure 2(c)), and the activity of elf18 to induce lesions was higher than that of flg22 (Figure S2(a)). These findings show that the leaf surface treatment with both tested peptide MAMPs derived from bacteria is sufficient to induce leaf lesions in the ns1-1 mutants, indicating a peptide MAMP-triggered cell death response in this mutant background. Next we assessed whether the fungus-derived MAMP chitin is involved in the Co-triggered lesion formation in the ns1-1 mutants of Col-0. Arabidopsis CERK1 receptor kinase is known to be critical for chitin recognition in Arabidopsis (Miyata et al., 2007; Wan et al., 2008). Thus, we generated the ns1-1 cerk1-2 double mutant and inoculated leaves with Co spores. This treatment still induced lesion development in the ns1-1 cerk1 mutant (Figure 2(d)), indicating that CERK1-dependent chitin recognition is not essential for the Co-triggered and ns1-1-dependent leaf cell death. It is possible that ns1-1-dependent cell death in response to Co or Cg is triggered via extracellular recognition of unknown MAMPs shared between these two Colletotrichum species.

NSL1 accumulates at the plasma membrane

To investigate both the expression pattern and subcellular localization of NSL1, we generated ns1-1-3 transgenic lines expressing GFP-fused NSL1 under the control of 1.9 kb 5′ regulatory sequences of NSL1. Expression of GFP–NSL1 fully suppressed the ns1-1 lesion phenotype upon both MAMP and pathogen treatments, indicating functionality of the GFP–NSL1 fusion protein (Figures 3(a) and S3(a)). GFP–NSL1 fluorescent signals were undetectable in leaf cells in the absence of MAMP; however, a green fluorescent signal was clearly detectable at the cell periphery upon flg22 application (Figure 3(b)). This suggests that the MAMP treatment confers an enhanced accumulation of NSL1. This conclusion was corroborated in a time-course experiment by western blot analysis using anti-GFP antibody and revealed increased GFP–NSL1 steady-state levels at 24 and 48 hours post treatment (hpt) prior to the manifestation of MAMP-induced cell death in the ns1-1 mutants (Figure 3(d)). The GFP–NSL1 signal at the cell periphery was confined to the plasma membrane when the transgenic lines carrying GFP–NSL1 were treated with flg22 or Co (Figures 3(b) and S3(b)), which is distinct from the GFP fluorescence signal detected in the cytoplasm and nuclei of leaf epidermal cells when GFP alone was expressed under the control of 5′ regulatory sequences of NSL1 (Figure S3(c)). These findings suggest preferential localization of NSL1 at the plasma membrane, which is further supported by a plasmolysis assay conducted with the GFP–NSL1 line (Figure 3(c)).

Natural genetic variation in Arabidopsis determines the ns1-1 phenotypic outputs

We found marked phenotypic differences between ns1-1-1 and ns1-1-2 mutants in accession No-0 and ns1-1-3 and ns1-1-4 mutants in accession Col-0 (Figure 4(a)). To clarify whether these differences result from mutant allele-specific differences or accession-dependent variation in genetic backgrounds, we crossed the ns1-1-2 mutant with WT Col-0. Nine of the 38 F2 progeny plants that were homozygous for the ns1-1-2 allele exhibited the spontaneous leaf lesion phenotype and dwarfism similar to the ns1-1-2 parent. However, the phenotype of other F2 siblings that were also homozygous for ns1-1-2 was indistinguishable from the WT parent (Figure 4(b)). Furthermore, when the ns1-1-2 F2 progeny lines displaying WT-like growth were treated with flg22, these plants developed local leaf lesions and were indistinguishable from ns1-1-4 mutants (Figure 4(c)). In reciprocal experiments we crossed the ns1-1-4 mutant with
WT No-0 and selected seven F2 progeny homozygous for the nsl1-4 allele and observed that one progeny exhibited stunted plant growth and spontaneous leaf lesions resembling the nsl1-1 and nsl1-2 mutants (Figure S4). Together these findings suggest that: (i) the mutations in At1g28380 (NSL1) produce distinct phenotypic outputs in Col-0 and No-0 genetic backgrounds; and that (ii) all four alleles have the potential to cause similar phenotypes, depending on the genetic background of the plants.

**flg22-induced PCD in the nsl1 mutants depends on SA signaling but not on reactive oxygen species (ROS) generation**

The dwarf and leaf lesion phenotypes of Arabidopsis nsl1 plants of the accession No-0 are partially suppressed by a mutation in SID2 (EDS16), which encodes an enzyme necessary for pathogen-induced SA biosynthesis (Wildermuth et al., 2001; Noutoshi et al., 2006). We asked whether the flg22-triggered cell death of the nsl1 mutants of Col-0 also requires SID2. We generated nsl1-4 sid2-2 double mutants and found that flg22 did not induce cell death in this line (Figure 5(a)). Similarly, sid2 abolished pathogen (Co)-induced cell death in the nsl1 background (Figure S5). The nsl1-dependent cell death was also reduced by a mutation in PAD4, which is involved in SA signaling (Jirage et al., 1999) (Figure S5). These findings indicate that the tested MAMPs trigger PCD in the nsl1 mutants of Col-0 via the SA pathway.

As cell death in plants is often linked to ROS production we examined hydrogen peroxide accumulation in nsl1 mutants treated with MAMPs. It is known that MAMP treatment causes rapid ROS production within minutes in the Arabidopsis WT plants. Here we tried to detect hydrogen peroxide (H2O2) accumulation by 3,3′-diaminobenzidine (DAB) staining (Thordal-Christensen et al., 1997) at later time point, i.e., 48 h after MAMP application (before lesions became visible in the nsl1-3 and nsl1-4 mutants). Upon flg22 treatment, H2O2 accumulation was found to be higher in nsl1 leaves than in WT plants, indicative of an enhanced ROS accumulation in nsl1 plants at 48 h after MAMP application (Figure 5(b)). Although the flg22-induced ROS accumulation in the nsl1 sid2 double mutant at 48 h was similar to that in nsl1 plants (Figure 5(b)), the flg22-induced lesion formation was strongly diminished in this double mutant (Figure 5(a)). This finding suggests that SA biosynthesis is not essential for the flg22-triggered H2O2 over-accumulation in the nsl1 mutants. We next generated and examined nsl1 rbohD double mutants. The increased H2O2 accumulation upon flg22 treatment at 48 h after MAMP application was abolished in this line (Figure 5(b)), indicating that the MAMP-induced ROS accumulation in the nsl1 mutants prior to the MAMP-induced cell death depends on the NADPH oxidase RBOHD (Torres et al., 2002). By contrast, the flg22-induced lesion formation in the nsl1 rbohD mutant was the same as in the nsl1 mutants (Figure 5(a)). Collectively, these results indicate that flg22 elicits cell death and increased ROS generation in the nsl1 mutants and that SID2-dependent SA biosynthesis, but not RBOHD-dependent ROS accumulation, is critical for the MAMP-triggered cell death.

As nsl1 plants exhibited a hydrogen peroxide over-accumulation phenotype and lesion formation upon flg22 treatment, we also tested nsl1 plants for other MAMP-triggered cellular responses. It is known that flg22 rapidly (within minutes) activates mitogen-activated protein kinases (MAPKs) such as MPK3 and MPK6 (Asai et al., 2002). We investigated this early MAMP response and found that the nsl1-3 and nsl1-4 mutations had no apparent effects on the timing of MAPK activation (Figure 5(c)). flg22 also induces PMR4/GSL5-dependent callose deposition in Arabidopsis (Jacobs et al., 2003; Nishimura et al., 2003; Clay et al., 2009). The nsl1-3 and nsl1-4 mutants accumulated callose upon flg22 treatment as did WT plants (Figure S6). Clay et al. (2009) reported that in Arabidopsis flg22-induced callose deposition is dependent on PEN2-mediated IG-metabolism. PEN2 encodes a myrosinase that hydrolyzes 4-

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Figure 2. MAMPs trigger programmed cell death in nsl1 plants.
(a) Bacterial peptide MAMP flg22 induces programmed cell death (PCD) in the nsl1-3 and nsl1-4 mutants. The peptide solution was dropped on the leaf surface of the tested Arabidopsis lines, and the treated lines were incubated for 4 days.
(b) flg22 induces PCD in cotyledons of the nsl1 seedlings. Arrows indicate cotyledons where flg22 solution was spotted.
(c) Bacterial peptide MAMP elf18 induces PCD in the nsl1 mutants.
(d) CERK1-dependent chitin recognition is not essential for Co-induced PCD in nsl1. Conidial suspension of Co was spray-inoculated on the tested lines, and the inoculated plants were incubated for 6 days.

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methoxyindol-3-ylmethyl glucosinolate (4MI3G) as in planta substrate for antifungal defense responses (Bednarek et al., 2009; Clay et al., 2009). We generated the nsl1-4 pen2 double mutant and confirmed that callose deposition in nsl1 was suppressed by the pen2 mutation (Figure S6). This suggests that there are no obvious effects of nsl1 on the flg22-induced callose deposition pathway. The nsl1 sid2 mutant accumulated callose upon flg22 treatment as did

Figure 3. flg22-Induced expression of NSL1 and its cellular localization.
(a) Expression of GFP-NSL1 under its native promoter complements the nsl1 phenotype. The flg22 (10 μM) solution was dropped on the leaf surface of the tested Arabidopsis lines, and the plants were incubated for 4 days.
(b) Localization of GFP-NSL1 under the MAMP treatment. flg22 was administered to the transgenic nsl1 line expressing GFP-NSL1 under its native promoter. A photograph was taken at 18 hpt of flg22. Bars = 50 μm. DW, distilled water was treated.
(c) Plasmolysis assay toward the transgenic line expressing GFP-NSL1. The nsl1 line expressing GFP-NSL1 was treated with flg22 for 24 h and subjected to the plasmolysis treatment. White dot lines represent boundaries of each epidermal cell. Bar = 30 μm.
(d) Immunoblot of GFP-NSL1 fusion protein probed with the anti-GFP antibody. Arrowhead indicates GFP-NSL1 protein. flg22 enhanced GFP-NSL1 accumulation at 24 and 48 hpt.

Figure 4. Physiological effects of the nsl1 insertion mutations are changed in the background of a distinct accession.
(a) The phenotype of the nsl1 mutants in No-0 background. Each plant was grown for 25 days without MAMP treatment.
(b) The phenotypic variation among F2 progenies carrying the nsl1-2 allele generated from cross between the nsl1-2 mutant and the wild-type (WT) Col-0. Among F2 progenies derived from cross of the nsl1-2 with Col-0, multiple nsl1-2 lines were identified. Although a part of the identified nsl1-2 F2 progenies exhibit the growth reduction with yellowish lesions in the absence of MAMP treatment (ex. progeny B), the other nsl1-2 F2 progenies did not exhibit any growth reduction and lesions the same as WT (ex. progeny A).
(c) flg22 treatment induced PCD in the nsl1-2 F2 progenies showing the WT growth phenotype. flg22 at 10 μM was spotted in each tested plant and treated plants are incubated for 4 days. The nsl1-2 F2 progenies showing the WT growth phenotype (progeny A) developed lesions on the flg22-treated area, which is quite similar to the phenotype of the nsl1-4 mutant of Col-0.
We monitored accumulation levels of camalexin and I3CAs in leaf extracts (Figure S8; Bednarek et al., 2009; Lu et al., 2015) (Figures 7(a) and S8). Accumulation of these compounds increased markedly in the nsl1-3 and nsl1-4 mutants as compared with Col-0 at both 48 and 72 h after flg22 application, whereas their low-level constitutive accumulation in nsl1 was comparable to that observed in WT (Figure 7(a)). These results indicate that the lack of functional NSL1 enhances the flg22-induced accumulation of PEN2 pathway end products, including RA, I3A and 4OGlcI3F upon flg22 treatment, which both increased markedly in the nsl1-3 and nsl1-4 mutants compared to WT (Figure 6(b)). We thus conclude that the CYP81F2/PEN2 pathway as well as the SA pathway is involved in the MAMP-triggered cell death in the absence of functional NSL1 protein.

To directly assess the activity of the PEN2-dependent metabolic pathway in nsl1 mutants, we monitored accumulation levels of its end products, (i) raphanusic acid (RA) and (ii) indol-3-ylmethyl glucosinolate (I3G), and (iii) 4-O-β-D-glucosyl-indol-3-yl formamide (4OGlcI3F) derived from 4-methoxy-indol-3-ylmethyl glucosinolate (4MI3G), in leaf extracts (Bednarek et al., 2009; Bednarek, 2012; Lu et al., 2015) (Figures 6(a) and S8). Accumulation of these compounds increased markedly in the nsl1-3 and nsl1-4 mutants as compared with Col-0 at both 48 and 72 h after flg22 application, whereas their low-level constitutive accumulation in nsl1 was comparable to that observed in WT (Figure 7(a)).

IGs, which are PEN2 substrates, are derived from Trp, an aromatic amino acid that in Arabidopsis serves as an precursor of other pathogen-inducible metabolites, including camalexin and indol-3-carboxylic acid derivatives (I3Cas) (Bednarek, 2012; Bednarek et al., 2005) (Figure S8). Camalexin is an antifungal metabolite, regarded as Arabidopsis phytoalexin, and is produced by a relatively well characterized pathway involving the cytochrome P450 monoxygenases PAD3 (Phytoalexin Deficient 3) (Zhou et al., 1999). We monitored accumulation levels of camalexin and I3Cas upon flg22 treatment, which both increased markedly in the nsl1 mutants compared to WT (Figure 7(a)).

The increased camalexin accumulation suggested that this antifungal metabolite might be linked to the flg22-induced PCD in the nsl1 plants. To test this we generated and examined nsl1 pad3 double and nsl1 pen2 pad3 triple mutants. The nsl1 pad3 line developed a cell death phenotype; however, leaf lesions were weaker in this line compared to nsl1 plants (Figure S9(a)). Although nsl1 pen2 leaves occasionally developed weak lesions upon flg22 treatment, the nsl1 pen2 pad3 triple mutant completely failed to mount a cell death response upon flg22 treatment.
These findings suggest that the phytoalexin camalexin is involved in the cell death of flg22-treated nsl1 mutants, although the PEN2-dependent metabolites play a major role. Taken together, our findings demonstrate that two branches of Trp-derived secondary metabolism together with SA biosynthesis are needed for MAMP-triggered lesion formation in nsl1 mutants.

Additionally, in contrast to nsl1 and nsl1 sid2 plants, ROS accumulation at 48 h after flg22 application was abrogated in the nsl1 pen2 and nsl1 pen2 pad3 plants (Figure 5(b)). The ROS accumulation in the nsl1 pad3 plants was similar to the nsl1 plants (Figure 5(b)). Thus, the enhanced accumulation of the Trp-derived and PEN2-dependent metabolites is involved in triggering ROS accumulation in the nsl1 mutants upon MAMP treatment, whereas the SA biosynthesis is not essential for this phenomenon.

**Increased accumulation of SA in nsl1 depends on Trp metabolism**

Next we examined the relationship between Trp metabolism and SA biosynthesis in nsl1-dependent leaf cell death. Although SA levels in non-challenged nsl1-3 and nsl1-4 leaves were similar to those in WT, SA accumulation in the nsl1 mutants at 24 h after flg22 application was increased compared to WT (Figure 7(b)). These results suggest that MAMP treatment triggered SA biosynthesis in nsl1 plants. Because nsl1 sid2 mutants fail to activate cell death upon flg22 treatment, the observed SA accumulation is needed for flg22-induced lesion formation in a nsl1 background. Remarkably, the pen2 pad3 double mutation caused complete abrogation of SA accumulation in the nsl1 background (Figure 7(b)). Together with our aforementioned genetic evidence, the finding suggests that Trp-derived metabolites are needed for SA accumulation in flg22-treated nsl1 mutants. These results reveal an unexpected molecular link between antimicrobial metabolites and the SA defense phytohormone pathway.

To further investigate this link, we tested the effects of the sid2 mutation on Trp metabolism in the nsl1 background. The accumulation of the end products of the PEN2 pathway (I3A and RA) in the nsl1 mutant was not decreased by the sid2 mutation (Figure 7(c)). By contrast, the elevated levels of camalexin and I3CAGlc were partially suppressed in the nsl1 sid2 plants (Figure 7(c)), implying the involvement of SA in the accumulation of these metabolites. As discussed above, PEN2 (but not PAD3) is critical for MAMP-triggered cell death in nsl1 plants (Figures 6 and S9). Collectively, these findings suggest that the increased accumulation of PEN2-related metabolites activates SA biosynthesis and signaling in the nsl1 plants of Col-0 upon MAMP treatment, which leads to a genetically determined cell death in this mutant.

**DISCUSSION**

Unlike ETI, MTI is normally uncoupled from host cell death responses. ETI displays amplified and sustained immune responses compared with MTI, suggesting that quantitative rather than qualitative differences account for the activation of ETI-associated cell death (Tsuda and Katagiri, 2010). However, it remains unclear whether an amplified MTI is able to trigger cell death via the same or different genetic programs as in ETI. Here we have shown that leaf surface treatment with peptide MAMPs induces a genetically defined cell death in the Arabidopsis nsl1 mutants (Col-0...
Figure 7. Accumulation of Trp-derived metabolites and salicylic acid in the nsl1 mutants sprayed with flg22 (10 μM).
(a) Accumulation of Trp-derived secondary metabolites at the indicate time points after flg22 treatment.
(b) Total salicylic acid levels in nsl1 mutants 24 h after flg22 application.
(c) The effect of sid2 mutation on accumulation of Trp-derived metabolites in the nsl1 background. The means and standard deviations (SDs) were calculated from three independent experiments. In each experiment single samples have been collected per genotype in each time point. The statistical significance of differences between means was determined on log-transformed data by Tukey test.
**P < 0.01, *P < 0.05 [comparison of Col-0 with nsl1 mutants in Figure 7(a, b); comparison of nsl1 mutants with nsl1 sid2 mutant in Figure 7(c)].
NT: nontreated control plants, I3A: indol-3-ylmethylamine; RA: raphanusamic acid; 4OGlcl3F: 4-O-β-D-glucosyl-indol-3-yl formamide. I3CAGlc: glucose ester of indole-3-carboxylic acid; 6OGlcl3CA: 6-O-β-D-glucosyl-indole-3-carboxylic acid, Ca: camalexin.

background) that are depleted in an MACPF domain protein, thereby revealing a potential MAMP-triggered PCD.

The relationship between MTI and ETI described above prompts the question whether MTI responses in nsl1 plants are amplified compared with those in WT. It has been reported that flg22 triggers the accumulation of SA as well as ROS in Arabidopsis WT (Tsuda _et al._, 2008). We found that the nsl1 mutations enhanced these MTI outputs (Figures 5(b) and 7(b)), supporting the notion that MTI responses are enhanced in nsl1 plants. We found that enhanced activation of SA biosynthesis is critical for MAMP-triggered PCD in nsl1 (Figure 5(a)). It has been reported that ETI, triggered by the bacterial effector AvrRpt2, accompanies sustained activation of MAPK signaling to regulate SA-responsive genes independently of SA, and this compensatory relationship between MAPKs and SA signaling is thought to confer robustness to ETI compared with MTI (Tsuda _et al._, 2013). In contrast, nsl1 plants did not exhibit sustained activation of MPK3 and MPK6 upon MAMP treatment, at least early after MAMP treatment (within 30 min; Figure 5(c)), suggesting that in the absence of NSL1 enhanced SA biosynthesis plays a major role for MAMP-triggered PCD without sustained MAPK activation.

Our genetic analysis demonstrated that MAMP-triggered PCD in nsl1 plants is not only dependent on SA biosynthesis, but also on Trp-derived metabolites (Figure 6). Further analyses suggested that an increased production of these secondary metabolites activated SA biosynthesis (Figure 7(b)), thereby revealing a previously unsuspected role of Trp-derived metabolites in the regulation of the SA defense phytohormone pathway and PCD. DAB staining assays indicated that flg22 triggers ROS generation in both nsl1 and nsl1 sid2 plants, but not in nsl1 pen2 plants, at
48 h after the treatment (Figure 5(b)). This suggests that the accumulation of PEN2-related metabolites in nsl1 plants is essential for MAMP-triggered PCD and that subsequent SA accumulation amplifies the PCD response.

Comparison of nsl1 pen2 with the nsl1 pad3 mutant showed that the PEN2-dependent products of IG hydrolysis play a major role in nsl1 PCD, whereas camalexin had a minor role (Figure S9). In this context it is notable that PEN2, but not PAD3, was previously shown to be required for flg22-induced callose deposition in Arabidopsis (Clay et al., 2009). We found that SA signaling is critical for flg22-induced PCD (Figure 5(a)), but not for flg22-triggered callose deposition in nsl1 plants (Figure S5). This finding suggests that PEN2-dependent metabolites can act as signal molecules that activate the SA pathway leading to PCD, especially in the absence of NSL1, probably uncoupled from the flg22-induced callose deposition pathway. Furthermore, a recent report suggested that PEN1, PEN2, and PEN3 contribute to effector-triggered PCD upon challenge with bacterial or oomycete pathogens, implying a general role of PEN2-dependent metabolites in defense-related PCD forms (Johansson et al., 2014). In contrast to the end products of the PEN2 pathway (JA and RA), the accumulation of camalexin and I3CAs in nsl1 plants was partially suppressed by sid2, suggesting that SA contributes to an increased production of these metabolites (Figure 7(c)). Similarly, the accumulation of camalexin, but not I3A and RA, is strongly induced by Cg invasion in Arabidopsis (Hiruma et al., 2013). These data point to a distinct regulatory relationship between the PEN2-related metabolic pathway and the camalexin/I3CA pathway.

We identified the nsl1 mutants by screening with C. oribiculare (C), a nonadapted pathogen on Arabidopsis (Figure 1(a)). In addition, we showed that a closely related nonadapted Colletotrichum species, C. gloeosporioides (Cg), also triggered PCD in nsl1 without successful fungal invasion (Figure 1(c, d)). The analysis on the nsl1 cerk1 mutants suggested that CERK1-dependent chitin recognition is not essential for Co-triggered PCD (Figure 2(d)). These findings indicate that Colletotrichum species have unidentified MAMP(s) that trigger PCD in the nsl1 mutants of Arabidopsis accession Col-0. We reported that entry attempts of Co and Cg are terminated in Arabidopsis Col-0 by pre-invasive nonhost resistance that requires CYP81F2 and PEN2 (Shimada et al., 2006; Hiruma et al., 2010). Thus, the pre-invasive nonhost resistance of Arabidopsis might be triggered by the same unidentified MAMP(s) that induces PCD in the nsl1 mutants. Consequently, the nsl1 mutants could be utilized as tool to identify the unidentified MAMP(s) of Colletotrichum species.

In the animal complement system, MACPF domain proteins are assembled to form pores in the membranes of invasive bacteria, which help to kill them as a part of the immune response (Rosado et al., 2008; Kondos et al., 2010; McCormack et al., 2013). In contrast, upon MAMP treatment, Arabidopsis NSL1 localizes to the plasma membrane of host cells without killing them (Figure 3b, c), implying that NSL1 has molecular functions distinct from those of the animal MACPF proteins in immunity or that pore formation requires an additional cue. The other regions of NSL1 are conserved in higher plants, but distinct from those of animal MACPF proteins (Gilbert et al., 2013). One possibility is that putative NSL1 pore formation is regulated by plant-unique regions flanking the MACPF domain or by other plant factors, similar to the inhibitory effect of CD59 on human MACPF proteins of the complement system (Huang et al., 2006). Here we indicate that the phenotypic difference of the nsl1-3 and nsl1-4 mutants (Col-0 background) from nsl1-1 and nsl1-2 mutants (No-0 background) depends entirely on natural genetic variation of other loci in their respective parental accessions and not on mutant allele-specific differences (Figures 4 and S4). Interestingly, the nsl1-4 mutation locates to the plant-unique region close to the C-terminus of NSL1, suggesting that this region is critical for protein function(s) (Figures 1(b) and S1). Also, the nsl1-3 mutation changes Gly to an Arg and this Gly residue is conserved in all Arabidopsis NSL1 homologs (At4 g24290, At1g29690, and At1g14780; Figure S1). These findings will be useful for future structural and functional analyses of plant MACPF domain proteins that are distinct from animal MACPF domain proteins.

Why do the nsl1 mutant alleles cause severe dwarfism phenotypes in the No-0 but not in the Col-0 genetic background? One possibility is that NSL1 is guarded by an unidentified intracellular NLR-type immune sensor present in No-0 and absent in Col-0. For example, the accelerated cell death 11 (acd11) ‘lesion mimic’ mutant of Arabidopsis exhibits autoimmune phenotypes such as constitutive defense responses and cell death without pathogen perception and dominant negative mutations in the TIR-type NLR LAZ5 suppress acd11 death (Palma et al., 2010). Similarly, a generic screen for suppressors of the autoimmune phenotype of Arabidopsis mkk1 mkk2 mutants, in which the MAMP-induced MAP kinase cascade is disrupted, revealed the NLR SUMM2 (Zhang et al., 2012). A truncated Arabidopsis NLR Protein, TIR-NBS2, was recently shown to be required for activated defense responses in the exo70B1 mutant, encoding an evolutionary conserved component of the exocyst complex that serves a critical role in plant secretory pathways (Zhao et al., 2015). Common features of ‘autoimmunity’ in nsl1, exo70B1, acd11 and mkk1 mkk2 plants are constitutive defense responses, dwarfism and/or leaf necrosis. However, mutations in RAR1 and SG71, which are needed for the function of a subset of NLR-type immune receptors, have no impact on the nsl1 phenotype of No-0 (Noutoshi et al., 2006; Shirasu, 2009). Also, there is a possibility that natural variation of genes involved in the
biosynthesis of IGs in Col-0 and No-0 accessions might affect the nsl1 phenotype. Interestingly, an insertion mutation in At1g29680, a close homolog of NSL1, resulted in growth retardation and spontaneous leaf cell death in the Col-0 background (Morita-Yamamuro et al., 2005), resembling the nsl1 mutants of No-0, and suggesting overall related but accession-dependent functions for each MACPF domain protein. These findings and the nsl1 mutant alleles described in the present study provide genetic tools and a genetic framework to examine in future work the biochemical function(s) of plant MACPF domain proteins in plant defense to pathogens.

EXPERIMENTAL PROCEDURES

Screening of Arabidopsis mutants that form lesions after C. orbiculare inoculation

We purchased EMS-mutagenized M2 seeds of Col-0 from Lehle Seeds (Round Rock, TX, USA). Plants approximately 4 weeks old were spray-inoculated with a conidial suspension (~5 × 10⁵ conidia/ml) of C. orbiculare (Co). Inoculated plants were placed in a growth chamber at 25°C with a cycle of 16 h of light and 8 h of dark and maintained at 100% relative humidity. The lesion phenotype of the inoculated M2 plants was investigated at 5–7 days post inoculation (dpi). Col-0 did not exhibit any lesions after the Co inoculation.

Plant lines and growth

Arabidopsis thaliana accession Col-0 was used as the WT. pen2-1, pen2-2 (Lipka et al., 2005), pen3-1 (Stein et al., 2006), cpy81F2-2 (Bednarek et al., 2005), atrbohD (Torres et al., 2007), sid2-2 (eds18) (Dewdney et al., 2000), pad3-1 (Hirano et al., 1999), pad4-1 (Glazebrook and Ausubel, 1994), nsl1-1 and nsl1-2 (Noutoshi et al., 2006) and cerk1-2 (Miyata et al., 2007) mutants were described earlier. Plants were sown on rockwool, treated at 4°C in the dark for 2 days, and grown at 25–26°C with 16 h of illumination per day in nutrient medium.

Fungal materials

The Co WT strain 104-T (MAFF 240422), a pathogen of cucumber, is a stock culture of the Laboratory of Plant Pathology, Kyoto University. Colletotrichum gloeosporioides S9275 (Cg), a pathogen of mulberry, was provided by Shigenobu Yoshida (National Institute for Agro-Environmental Sciences, Japan). Cultures of the fungal isolates were maintained on 3.9% (w/v) potato dextrose agar medium (PDA; Difco, Detroit, MI, USA) at 24°C in the dark. Conidia of Cg were prepared from fungal culture grown with a cycle of 16 h of black light and 8 h dark.

MAMP treatment

The MAMP peptide dissolved in water was administered to Arabidopsis plants by spotting 5-μL drops on each leaf. Treated plants were then placed in a growth chamber at 25°C with a cycle of 16 h of light and 8 h of dark, and maintained at 100% relative humidity.

Generation of double or triple mutants by crossing

By crossing nsl1-3 and nsl1-4 with the mutants described above, we generated double or triple mutants as following: nsl1-3 cerk1-2, nsl1-4 sid2-2, nsl1-3 rbohD, nsl1-3 pad4-1, nsl1-3 pen2-1, nsl1-4 pen2-1, nsl1-3 pen2-2, nsl1-3 cpy81F2-2, nsl1-4 pad3-1, and nsl1-4 pen2-1 pad3-1. All of the mutant lines except pen2-1 (Col-0 gl1 background) were in the Col-0 background. The nsl1-2 mutants (No-0 background) were crossed with Col-0 WT. The nsl1-4 mutant (Col-0 background) was also crossed with No-0 WT. The nsl1-3, nsl1-4, pen2-1, pen3-1, and pad4-1 mutations were checked with the corresponding specific primers for derived cleaved amplified polymorphic sequence (dCAPS) markers found by the dCAPS Finder 2.0 (http://helix.wustl.edu/dcaps/dcaps/dcaps.html). Both types of polymerase chain reaction (PCR) products (WT and mutant) were cleaved with corresponding enzymes. The pen2-2, cpy81F2-2, atrbohD and the nsl1-2 mutants had T-DNA or transposon insertions, and the insertions were checked by PCR using corresponding gene-specific primers and the insertion type-specific primers. The fast neutron-generated sid2-2 mutation was also checked by corresponding specific primers. The pad3-1 mutation was checked by sequencing using the listed primers. Table S1 shows a list of primers used in genotyping the mutations.

Analyses of Trp metabolites and salicylic acid

Plant samples (100–200 mg fresh weight for each sample) were collected at 0, 48, and 72 h (Trp-metabolites) or 24 h (SA) after spray inoculation with flyg22 (10 μM) and frozen in liquid nitrogen. Extraction and HPLC analysis of Trp derivatives was performed as previously described (Bednarek et al., 2009; Lu et al., 2015). Extraction and analysis of total SA followed the protocol of Bartsch et al. (2006). Each line was tested in three independent experiments. In each experiment single samples have been collected per genotype in each time point.

Experimental methods for DAB staining, confocal microscopy, TILLING, trypan blue staining, western blot analysis, assays of MAMP-induced responses, and plasmid construction are described in Methods S1.

ACKNOWLEDGEMENTS

We thank Jeffery Dangl (University of North Carolina, USA: atrbohD), Volker Lipka (Georg-August-University Goettingen, Germany, sid2-2), Takashi Kuromori (RIKEN CSRS, Japan, nsl1-1 and nsl1-2), Naoto Shibuya (Meiji University, Japan, cerk1-2), the ABRC (Ohio State University, USA: pad3-1, pad4-1) for providing seeds. This work was supported in part by Grants-in-Aid for Scientific Research (15H05780, 15H04457 and 20380027) from the Ministry of Education, Culture, Sports, Science and Technology of Japan; by Asahi Glass Foundation, and by the Science and Technology Research Promotion Program for Agriculture, Forestry, Fisheries and Food Industry (to Y.T.). Work in the Pawel Bednarek laboratory was supported with an EMBO Installation Grant. We thank Mika Kojima, Hiroe Utsubi and Malgorzata Zielinska for technical assistance.

CONFLICT OF INTEREST

The authors have no conflict of interests to declare.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Alignments of the A. thaliana NSL1 sequence with those of the other three proteins of A. thaliana having a MACPF domain.

Figure S2. Application of 1 μM flyg22 and elf18 on the nsl1 plants.
Figure S3. Analysis of the transgenic nsl1 lines expressing GFP-NSL1 where Co was inoculated.

Figure S4. The nsl1-4 mutation causes the growth retardation and spontaneous necrotic lesions in Arabidopsis plants.

Figure S5. Mutations of genes involved in SA signaling reduced the nsl1-PCD under the both MAMP and pathogen treatments.

Figure S6. fig22-triggered callose deposition is not altered by the nsl1 mutation.

Figure S7. pen2 mutations suppressed the nsl1-PCD under the pathogen treatment.

Figure S8. A model of Trp metabolisms pathways involving PEN2, CYP81F2 and PAD3.

Figure S9. Contribution of camalexin to fig22-triggered PCD.

Table S1. Primers used in this study.

Methods S1. Supplementary methods.

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