A novel allele of the Arabidopsis thaliana MACPF protein CAD1 results in deregulated immune signaling

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Abstract

Immune recognition in plants is governed by two major classes of receptors: pattern recognition receptors (PRRs) and nucleotide-binding leucine-rich repeat receptors (NLRs). Located at the cell surface, PRRs bind extracellular ligands originating from microbes (indicative of "non-self") or damaged plant cells (indicative of "infected-self"), and trigger signaling cascades to protect against infection. Located intracellularly, NLRs sense pathogen-induced physiological changes and trigger localized cell death and systemic resistance. Immune responses are under tight regulation in order to maintain homeostasis and promote plant health. In a forward-genetic screen to identify regulators of PRR-mediated immune signaling, we identified a novel allele of the membrane-attack complex and perforin (MACPF)-motif containing protein CONSTITUTIVE ACTIVE DEFENSE 1 (CAD1) resulting from a missense mutation in a conserved N-terminal cysteine. We show that cad1-5 mutants display deregulated immune signaling and symptoms of autoimmunity dependent on the lipase-like protein ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1), suggesting that CAD1 integrity is monitored by the plant immune system. We further demonstrate that CAD1 localizes to both the cytosol and plasma membrane using confocal microscopy and subcellular fractionation. Our results offer new insights into immune homeostasis and provide tools to further decipher the intriguing role of MACPF proteins in plants.

Keywords: Arabidopsis thaliana; MAMP; CAD1; immunity; membrane-attack complex; perforin

Introduction

Mechanisms to sense and respond to pathogens have evolved in all forms of life. Eukaryotic cells contain different types of receptors that detect the presence of microbes and activate immune signaling cascades resulting in cellular reprogramming. In plant cells, recognition of pathogens is achieved through two major groups of receptors: those on the cell surface and those located intracellularly (Jones and Dangl 2006, Couto and Zipfel 2016). Receptors at the cell surface are known as pattern recognition receptors (PRRs), which bind conserved features of entire groups of microbes known as microbe-associated molecular patterns (MAMPs). MAMPs are typically integral to microbial lifestyles and are therefore under strong selective pressure (McCann et al. 2012). Classic examples include bacterial flagellin and Elongation Factor-Thermo Unstable (EF-Tu) (Couto and Zipfel 2016). In the model plant Arabidopsis thaliana, flagellin (or the minimal epitope flg22) is recognized by its cognate PRR FLAGELLIN SENSING 2 (FLS2), while EF-Tu (or the minimal epitope elf18) is recognized by EF-Tu RECEPTOR (EFR) (Couto and Zipfel 2016). PRR surveillance can be thought of as detection of "non-self" that can perceive both pathogenic and non-pathogenic microbes (Couto and Zipfel 2016). Endogenous “damaged self” molecules, such as cell wall fragments or peptides released during cell damage are similarly recognized by PRRs (Segonzac and Monaghan 2019). For example, the peptide AtPep1 binds and activates the receptors FEP-RECEPTOR 1 (FEP1) and FEP2, triggering classical immune responses (Couto and Zipfel 2016). PRRs have been shown to function in complex with coreceptors or auxiliary proteins (Macho and Zipfel 2014; Couto and Zipfel 2016). One example of a co-receptor is BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED KINASE 1 (BAK1), that forms complexes with several PRRs including FLS2, EFR, and FEP1/2. PRR complex activation triggers an influx of calcium ions, an apoplastic oxidative burst, and phosphorylation-mediated kinase cascades leading to transcriptional reprogramming and basal immune responses (Couto and Zipfel 2016).
In order to successfully colonize plants, adapted pathogens have evolved ways to secrete effector proteins into plant cells to suppress immune responses (Dou and Zhou 2012). In the evolutionary “arms race” that exists between hosts and their pathogens, resistant plants have evolved intracellular receptors to intercept effectors and overcome disease. Most intracellular immune receptors contain an internal nucleotide-binding region (NB) and C-terminal leucine-rich repeats (LRR), preceded by an N-terminal domain that contains either a coiled-coil (CC) or Toll/Interleukin-1 receptor (TIR) region (Jones and Dangl 2006). The family is known as NB-LRR receptors (or NLRs), which are typically classed as either CC-NB-LRRs (CNLs) or TIR-NB-LRRs (TNLs). NLRs of both classes scan for the presence of pathogenic effectors, either through direct protein binding or by “guarding” the state of effector targets (van der Hoorn and Kamoun 2008). Activation of NLRs triggers the production of reactive oxygen species (ROS), activation of mitogen-activated protein kinases (MAPKs), and a sharp increase in the phytohormone salicylic acid (SA) that eventually results in a programmed cell death known as the hypersensitive response (HR) (Jones and Dangl 2006). Recent work has demonstrated that activated NLRs can form heteromeric “resistosomes” similar to “inflammasomes” in animals (Dangl and Jones 2019; Tian and Li 2020). For example, the CNL HOPZ-ACTIVATED RESISTANCE 1 (ZAR1) assembles into a pentameric ring-shaped complex predicted to facilitate lysis by forming pores in the plasma membrane (Wang et al. 2019a, b), and the TNLs RESISTANCE TO PERONOSPORA PARASITICA 1 (RPP1) and RECOGNITION OF XOPQ 1 (ROQ1) assemble into tetrameric clover-shaped holo-NADases (Ma et al. 2020; Martin et al. 2020). This localized defense response is accompanied by the release of systemic signals that result in broad-spectrum resistance in distant tissues (Jones and Dangl 2006).

While the HR can be effective at containing an infection, mechanisms that result in microbial death remain largely unknown. An increase in antimicrobial compounds such as ROS, SA, and nitric oxide hamper pathogen growth, but few molecular “agents of defense” have been uncovered in plants. One component of the mammalian immune system employs pore-forming proteins to directly target and lyse microbial membranes to clear infection (Rosado et al. 2007). Examples include perforin (PF), which can form oligomeric rings in target membranes, as well as complement proteins C5, C6, C7, C8, and C9, which together form a heteromeric cylindrical ring known as the membrane attack complex (MAC) (Bayly-Jones et al. 2017). Interestingly, pore-forming proteins are also an important aspect of pathogen virulence (Rosado et al. 2007). The membrane-attack complex and perforin (MACPF) signature motif Y/S-G-T/S-H-X7-G-G, in which X can be any amino acid, is found in proteins across kingdoms, including plants (Ni and Gilbert 2017; Yu et al. 2020).

There are four MACPF-motif containing proteins encoded in the model plant A. thaliana. Although two of these, CONSTITUTIVE ACTIVE DEFENSE 1 (CAD1) and NECROTIC SPOTTED LESIONS 1 (NSL1), have been studied genetically (Morita-Yamamuro et al. 2005; Noutoshi et al. 2006; Tsutsui et al. 2006; Asada et al. 2011; Fukunaga et al. 2017; Chen et al. 2020), the molecular function of Arabidopsis MACPF proteins remains elusive. In this study, we isolated a novel allele of CAD1 from a forward-genetics screen (Monaghan et al. 2014) designed to identify loci important for immune homeostasis. We show that immune signaling is deregulated when CAD1 is unstable and provide evidence that the N-terminus of CAD1 is particularly important for its integrity. Using confocal microscopy and subcellular fractionation, we show that CAD1 localizes to both the cytosol and the plasma membrane. Overall, our work confirms and builds on earlier observations and provides new insight into the biological function of MACPF proteins in plants.

Materials and methods

Plant materials and growth conditions

A. thaliana plants of the ecotypes Columbia-0 (Col-0) and Nossen-0 (N-0) were grown either on soil as one plant per pot in controlled environment chambers maintained at 20–22°C with a 16-h photoperiod. Isolation of modifier of bak1-5 (mob) mutants and Illumina sequencing of bulked segregants was described previously (Etherington et al. 2014; Monaghan et al. 2014). The bak1-5 mob4 mutants were purified by one backcross to bak1-5. Single cad1-5 mutants were obtained by crossing bak1-5 mob4 to Col-0. Null alleles cad1-2 (GABI_192A09), cad1-3 (GABI_385H08), and ns1-1 (PSH_21828) were obtained through the Nottingham Arabidopsis Stock Centre (NASC). Due to seedling necrosis, these lines had to be propagated as heterozygotes. Other mutant lines were described previously and genotyped to confirm homozygosity: bak1-5 (Schwessinger et al. 2011), Col eds1-2 (Feys et al. 2005), and ndr1-1 (Century et al. 1997). Double mutants were obtained by crossing homozygous plants and genotyping F2 segregants by allele-specific PCR. Homozygous doubles were propagated and confirmed in the F3 generation. Transgenic lines were generated by floral dip using Agrobacterium tumefaciens strain GV3101 according to standard protocols (Bent 2006). Only transgenic plants with single inserts, as indicated by 3:1 segregation on selection lines in the T2, were used for further analysis. Homozygous lines were selected based on appropriate antibiotic or herbicide resistance in the T3 generation.

Nicotiana benthamiana plants were grown as one plant per pot in controlled environment chambers maintained at 20–22°C with a 16-h photoperiod. Transient transformation using Agrobacterium tumefaciens strain GV3101 was conducted on plants that were between 5- and 6-week-old as described previously (Monaghan et al. 2014). All constructs were co-infiltrated with the silencing suppressor P19. Aggermoplast, primers, and constructs used in this study are listed in Supplementary Table S1.

Molecular cloning

For trans-complementation of bak1-5 mob4, a Gateway-compatible full-length genomic gCAD1 (At1g29690) fragment containing the native pCAD1 promoter [1027 bp upstream of the translational start codon, containing three predicted W-box cis elements as described in (Tsutsui et al. 2006)] and including the endogenous stop codon was amplified from Arabidopsis Col-0 genomic DNA using Phusion Taq polymerase (New England Biolabs) and cloned into pENTR using the D/Topo kit (Invitrogen). This was followed by recombination into pGW1_B (Nakagawa et al. 2007) by LR Clonase II (Invitrogen). Additional clones harboring CAD1 or NSL1 were designed and constructed for different end-uses as outlined in Supplementary Table S1. All constructs were verified by Sanger sequencing (The Centre for Applied Genomics, Toronto, or The Genome Analysis Centre, Norwich).

Immunity assays

Immunogenic peptides fg22, elf18, and AtFep1 were synthesized by EZ Biotech (Indiana USA). MAMP-induced ROS burst and seedling inhibition were performed as previously described (Bredow et al. 2019). Syringe-inoculations with Pseudomonas syringae pv.
tomato (Pto) DC3000 were done as previously described (Monaghan et al. 2009).

RNA extractions and real-time quantitative PCR
RNA extractions were either performed using TRI reagent (Sigma), as described previously (Monaghan et al. 2014), or using the Aurum Total RNA Mini Kit following manufacturer’s directions (Bio-Rad). CDNA was synthesized using Superscript III (Invitrogen) and quantitative PCR was performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) using Sso Advanced Universal SYBR Green Supermix (Bio-Rad) following manufacturer’s directions at 4°C. The total microsome preparations were isolated as described previously (Takahashi et al. 2014). Briefly, approximately 40–50 g (fresh weight) of above ground biomass was collected from 5-week-old Arabidopsis plants grown on soil. Leaf tissue was cut into small pieces in 400–500 mL of pre-chilled homogenizing medium [0.5 M sorbitol, 50 mM MOPS-KOH (pH 7.6), 5 mM EDTA (pH 8.0), 5 mM EDTA (pH 8.0), 1.5% (w/v) PVP-40, 0.5% (w/v) BSA, 2.5 mM PMSF, 4 mM SHAM, 2.5 mM DTT]. Tissue was then homogenized using a polytron generator (PT10SK, Kinematica Inc., Lucrene, Switzerland) and filtrates were collected by sieving the homogenate through Mericloth and removing cellular debris by centrifugation at 4°C (10,000 × g for 15 min). Heavy membrane fractions were collected by ultracentrifugation at 231,000 × g for 50 min and resuspended in 1 mL of microsome suspension (MS) medium [10 mM KH2PO4/K2HPO4 (K-P) buffer (pH 7.8), 0.3 M sucrose] with a teflon-glass homogenizer. Samples were centrifuged again at 231,000 × g for 50 min and the pellet was suspended in MS suspension medium (2 mL) prior to homogenization using an electric teflon-glass homogenizer. Soluble protein was collected by grinding ~500 mg of leaf tissue under liquid N2. Ground tissue was resuspended in 500 mL of native protein extraction buffer [10 mM Tris-HCl (pH 7.5), 25 mM NaCl] and incubated at 4°C for 1 h, with shaking. Lysates were centrifuged at 12,000 × g for 10 min (4°C) to remove cellular debris. Protein concentration was determined using Pierce Protein Assay Kit following the manufacturer’s instructions (ThermoFisher Scientific) and normalized to ~2 μg/μL.

Immunoblots
Immunoblots were performed according to standard protocols using polyclinidene difluoride membranes (Bio-Rad) blocked with 5% non-fat powdered milk in Tris-buffered saline containing 0.05–0.1% Tween-20. BAK1-FLS2 co-IP blots were probed with α-BAK1 (Schulze et al. 2010) or α-FLS2 (Gimenez-Ibanez et al. 2009) followed by α-rabbit-TrueBlot-HRP (eBioscience). CAD1 accumulation blots were probed with primary α-GFP (Roche) and secondary α-mouse-HRP (Sigma Aldrich). Subcellular fractionation blots were probed with primary α-H+-ATPase (Agrisera Antibodies) and secondary α-rabbit-HRP (Sigma Aldrich), or α-CtBPase (cytosolic fructose-1,6-biphosphatase) (Agrisera Antibodies) and secondary α-mouse-HRP (Sigma Aldrich). Blots were imaged by enhanced chemiluminescence (ECL) using Clarity Substrate (Bio-Rad) or ECL Prime (GE Healthcare) on a Chemidoc Imager (Bio-Rad) or using an X-Ray film developer.

Confocal microscopy
Fluorescent proteins and dyes were excited using a 488 Argon laser and collected using separate tracks to detect different emission wavelengths; GFP or YFP emission was collected at 510–540 nm; SynaptroEd/FM4-64 at 635–680 nm; chlorophyll autofluorescence at 680–700 nm. Images were taken using the Zeiss LSM710 confocal microscope in the Biology Department at Queen’s University.

Statistics
GraphPad Prism 8 was used to perform statistical tests on all quantitative data.

Accession numbers
CAD1 (At1g29560); NSL1 (At1g28380); BAK1 (At4g33430); EDS1 (At3g48090); NDR1 (At3g20600).

Data availability
Strains and plasmids are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables. Supplemental Material available at figshare: https://doi.org/10.25386/genetics.13724035.

Results and discussion
Isolation of a novel CAD1 allele that affects its protein abundance
The C408Y variant of the co-receptor BAK1 resulting from the bak1-5 mutation strongly impairs signaling mediated by multiple PRR receptor complexes (Schwessinger et al. 2011; Roux et al. 2011). Prolonged exposure to flg22, elf18, or AtPep1 results in impaired growth of wild-type seedlings, likely the result of sustained immune signaling over-ruling normal growth and development. However, seedling inhibition is partially blocked in bak1-5 mutants (Schwessinger et al. 2011; Roux et al. 2011). Although strongly reduced, immune responses are not completely blocked in bak1-5 mutants, providing a useful immune-deficient background in which to perform a forward-genetic screen. To identify novel regulators of immunity, we previously isolated modifier of bak1-5 (mob) mutants that restore immune signaling in bak1-5 (Monaghan et al. 2014; Stegmann et al. 2017). Here, we describe mob4, which partially restores the flg22-, elf18-, and AtPep1-triggered ROS production and seedling inhibition in bak1-5 (Figure 1A, Supplementary Figure S1).

F1 progeny from bak1-5 mob4 backcrossed to bak1-5 were insensitive to elf18 in seedling growth inhibition assays. Analysis of F2 progeny from the same cross indicated that mob4 is caused by a single, recessive locus (177/799 plants had regained elf18-triggered seedling growth inhibition; x² = 3.455; P = 0.0631). To identify the causative mutation in bak1-5 mob4, we bulked the elf18-sensitive F2 progeny and sequenced the mutant genome using the Illumina HiSeq platform. We identified unique single nucleotide polymorphisms (SNPs) in the bak1-5 mob4 genome by comparing the mutant genome to the parental bak1-5 genome that we previously sequenced (Monaghan et al. 2014). We used the CandiSNP web application (Etherington et al. 2014) to plot the
genomic positions of unique SNPs identified in bak1-5 mob4 with an allele frequency over 75%. This analysis clearly indicated linkage on the top arm of Chromosome 1 (Supplementary Figure S2A). We used a subset of these SNPs as markers and could further refine this region between 9.43 Mbp and 10.8 Mbp after conducting linkage analysis in individual F3 backcrossed lines. Contained within this region were single non-synonymous cytosine-to-thymine (or the complement; guanine-to-adenine) transitions in six genes (Supplementary Figure S2B). In particular, we found a mutation causing a cysteine-to-tyrosine substitution at position 43 in the MACPF protein CAD1 (Figure 1B). To confirm that this mutation was causative of the mob4 phenotype, we stably transformed bak1-5 mob4 with full-length genomic CAD1 and found that elf18-triggered ROS production (Figure 1A) was fully complemented in two independent homozygous bak1-5 mob4/pCAD1:gCAD1 lines. This confirmed that the mutant variant CAD1C43Y is responsible for mob4 phenotypes. We thus renamed this allele cad1-5 in accordance with previously isolated cad1 alleles (Morita-Yamamuro et al. 2005; Tsutsui et al. 2006; Asada et al. 2011; Chen et al. 2020).

Previous work demonstrated severe constitutive cell death in a complete loss-of-function cad1-1 allele (Morita-Yamamuro et al. 2005). We obtained additional null alleles, cad1-2 and cad1-3, and found that they similarly resulted in stunted growth and seedling lethality (Figure 1, C–D). We noted that bak1-5 cad1-5 plants have smaller rosettes than both Col-0 and bak1-5 plants, and develop necrotic lesions, particularly in older leaves (Supplementary Figure S3A). The degree of necrosis varied depending on environmental growth conditions, but bak1-5 cad1-5 plants were always able to complete their life cycle and set plenty of seed. This led us to hypothesize that cad1-related growth defects and cell death might be suppressed by bak1-5, which could explain why we
recovered a recessive cad1 allele from the mob screen. To test this, we crossed bak1-5 cad1-5 with Col-0 to isolate single cad1-5 mutants. We found that cad1-5 mutants looked phenotypically similar to bak1-5 cad1-5 (Supplementary Figure S3A). These results rather suggest that the CAD1^{C43Y} variant expressed in cad1-5 (Supplementary Figure S3B) is partially non-functional and does not cause as severe phenotypes as the complete loss of CAD1 observed in null mutants (Figure 1D).

Cysteines contain sulfur atoms in their R groups that are capable of forming disulphide bonds within a polypeptide chain and are therefore important for tertiary folding and protein stability. To test if the C43Y mutation affects CAD1 stability, we transiently expressed cauliflower mosaic virus (CaMV) 35S promoter-driven 35S:CAD1-GFP and 35S:CAD1^{C43Y}-GFP in Nicotiana benthamiana and found that while both variants migrated to the expected size of ~90 kDa in western blots, accumulation of CAD1^{C43Y} was clearly reduced (Figure 1E). This suggests that C43 is important for CAD1 stability, which likely explains the partial loss-of-function phenotypes in cad1-5 compared to null alleles.

Interestingly, a multiple sequence alignment of 523 MACPF-motif containing proteins encoded across land plants revealed that C43 is found within a conserved N-terminal motif G-x-G/F/Y-D-x-x-x-D-x-R-L-x-x-C-K (Figure 1F), underscoring the possibility that this residue may be important for the stability of MACPF-containing proteins more broadly.

### Reduced CAD1 accumulation correlates with enhanced immune signaling

Equipped with a genetic resource with which to assess the biological function of CAD1 without the complications associated with null alleles, we sought to assess immune responses in cad1-5. Being unable to perform assays alongside cad1 null-mutants, we generated two independent cad1-5/pCAD1: gCAD1 rescue lines to confirm that any phenotypes we observed were indeed caused by the cad1-5 mutation (Figure 2A). Similar to what has been reported for cad1 null alleles (Morita-Yamamuro et al. 2005; Asada et al. 2011), we found that cad1-5 plants displayed heightened basal expression of the immune marker gene PR1.
(Figure 2B), which was complemented in the two cad1-5/pCAD1:gCAD1 lines. We next assessed pattern-triggered immune responses and found that cad1-5 displayed enhanced elf18- and AtPep1-triggered ROS production (Figures 2C, Supplementary Figure S4A) and seedling growth inhibition (Figure 2D, Supplementary Figure S4B), which were again fully complemented in the cad1-5/pCAD1:gCAD1 lines. Notably, we also found that cad1-5 seedlings display extreme cell death when grown in the presence of elf18 (Supplementary Figure S4C), and develop necrotic lesions 24 h after infiltration with elf18 as adult plants (Supplementary Figure S4D).

A previous study (Morita-Yamamuro et al. 2005) reported enhanced resistance to the virulent bacterial pathogen Pseudomonas syringae pv. tomato (Pto DC3000) in cad1-1; however, plants of this genotype display extreme necrosis, making interpretation of these data difficult. Follow-up experiments in which CAD1 was transiently silenced using a dexamethasone-inducible construct confirmed that lower levels of CAD1 result in enhanced resistance to Pto DC3000 (Asada et al. 2011). We also syringe-inoculated plants with Pto DC3000, and similarly found that cad1-5 mutants harbored 10-fold less bacteria compared to Col-0 3 days after inoculation, which was again complemented in the cad1-5/pCAD1:gCAD1 transgenic lines (Figure 2E). To assess whether enhanced immune responses in cad1-5 could be due to constitutive or enhanced FRR complex formation, we tested the flg22-induced association between the FRR FLS2 and its co-receptor BAK1. Co-immunoprecipitation (co-IP) assays using native α-BAK1 and α-FLS2 antibodies demonstrated similar complex formation in Col-0 and cad1-5 (Supplementary Figure S4E), indicating that autoimmunity in cad1-5 is not caused by enhanced FLS2-BAK1 complex formation.

**Autoimmunity in cad1-5 is EDS1-dependent**

The null cad1-1 mutant accumulates high levels of the immune hormone SA (Morita-Yamamuro et al. 2005). Transgenic expression of bacterial NahG, an enzyme that degrades SA, suppressed seedling cell death in cad1-1 (Morita-Yamamuro et al. 2005)—a classical hallmark of deregulated NLR-mediated signaling (Rodriguez et al. 2016). If CAD1 is an integral component of the plant immune response, it may be guarded by an NLR; inappropriate perturbation of CAD1 may thus activate NLR signaling resulting in uncontrolled cell death via the HR (Rodriguez et al. 2016). NLRs can generally be classified into two groups: TNLs and CNLs (McHale et al. 2006), and it is possible to genetically manipulate components required for signaling through either class to delineate which type of NLR may be activated (Aarts et al. 1998). TNL signaling typically requires a family of nucleo-cytoplasmic lipase-like proteins including ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1), PHYTOALEXIN DEFICIENT 4 (PAD4), and SENESCENCE ASSOCIATED GENE 101 (SAG101) (Lapin et al. 2020), while CNL signaling typically requires the membrane-localized protein NON-RAcE SPECIFIC DISEASE RESISTANCE 1 (NDR1) (Century et al.1997; Coppening et al. 2004). With this in mind, we generated cad1-5 edr1-2 and cad1-5 nrd1-1 double mutants to interfere with TNL- and CNL-triggered signaling pathways in cad1-5, respectively. We found that the small size and necrosis present in cad1-5 plants was completely suppressed in cad1-5 edr1-2 but not in cad1-5 nrd1-1 (Figure 3A). Enhanced steady-state expression of PR1 was similarly suppressed in cad1-5 edr1-2, but not cad1-5 nrd1-1 (Figure 3B). We were next interested to test if the enhanced pattern-triggered responses and immunity in cad1-5 were dependent on EDS1. Interestingly, although the heightened elf18-triggered ROS production (Figure 3C) was restored in cad1-5 edr1-2, elf18-triggered seedling growth inhibition and cell death was not restored in cad1-5 edr1-2 (Figure 3D). In addition, we found that resistance to Pto DC3000 was suppressed in cad1-5 edr1-2 (Figure 3E). Although enhanced susceptibility in edr1-2 mutants (Aarts et al.1998) could confound our interpretation, we propose that most of the enhanced immune phenotypes observed in cad1-5 are dependent on EDS1-mediated signaling. EDS1 forms distinct signal-competent heteromeric complexes with SAG101 and PAD4 (Lapin et al. 2019). Interestingly, previous work indicated that pad4 was unable to suppress most cad1-related immune phenotypes (Tsutsumi et al. 2008), likely also requiring helper NLRs such as N REQUIRED GENE 1 (NRG1) (Lapin et al. 2019, 2020). Altogether, these genetic analyses suggest that autoimmunity in cad1-5 may be linked to activation of a TNL receptor; although this remains to be experimentally validated. Interestingly, CAD1 was recently suggested to play a role in maintaining above-ground microbiota diversity, as cad1 mutants present symptoms of dysbiosis (Chen et al. 2020). As high levels of SA in cad1 may confound this interpretation, cad1-5 edr1-2 might be an important tool to further dissect how CAD1 contributes to the maintenance of a healthy endophytic population.

CAD1 belongs to a small family of MACPF proteins in Arabidopsis. Included in this family are NSL1 (Noutoshi et al. 2006), and as-yet-uncharacterized proteins encoded by At1g14780 and At4g24290 (Morita-Yamamuro et al. 2005). Interestingly, loss-of-function ns1-1 mutants in the No-0 ecotype display spontaneous cell death similar to cad1 mutants (Noutoshi et al. 2006). Null ns1-3 mutants in the Col-0 ecotype do not suffer from autoimmunity as in No-0, but do display runaway cell death when inoculated with the non-adapted fungal pathogen Colletotrichum orbiculare or when treated with the MAMPs flg22 or elf18 (Fukunaga et al. 2017). Both autoimmune cell death in ns1-1 and induced cell death in ns1-3 are suppressed in ns1-1 eds1-61 (Noutoshi et al. 2006) and ns1-3 sid2 (Fukunaga et al. 2017), which cannot produce SA due to the loss of isochorismate synthase encoded by SID2/ICS1/EDS16 (Wildermuth et al. 2001). Furthermore, hyperactive immunity is suppressed in ns1-3 pad4, suggesting that NSL1 may also be guarded by a TNL (Fukunaga et al. 2017). Enhanced MAMP-induced immune responses in ns1 could also be suppressed by loss of PENETRATION 2 (PEN2), a myrosinase involved in the synthesis of tryptophan-derived indole glucosinolates (Fukunaga et al. 2017). Future analysis of cad1 pen2 mutants could determine if the loss of either or both CAD1 and NSL1 result in an increase of SA biosynthesis and tryptophan-derived secondary metabolites.

**CAD1 localizes to the plasma membrane and the cytosol**

Perforin and complement proteins are targeted to non-autonomous cellular membranes to create pores and lyse those cells (Rosado et al. 2007). While it is possible that CAD1 and related proteins are deployed to pathogen membranes similar to their mammalian homologs, we could not detect secretory signal peptides in the amino-acid sequences of Arabidopsis MACPF proteins using the SignalP server (Emanuelsson et al. 2007). Previous work showed that NSL1 accumulates at the plasma membrane following MAMP induction in complementing, native-promoter driven and N-terminally tagged ns1-3/pNSL1-GFP-cNSL1 lines in the Col-0 ecotype (Fukunaga et al. 2017). During the course of our studies, we generated CaMV 35S promoter-driven and C-terminally tagged ns1-1/35S::cNSL1-YFP lines in the No-0 ecotype to similarly test sub-cellular localization of NSL1. Stunted growth and spontaneous necrosis in ns1-1 were complemented in...
two independent homozygous transgenic lines (Supplementary Figure S5A), indicating functionality of the NSL1-YFP protein. We found that 35S-driven NSL1-YFP localized to the plasma membrane even in the absence of MAMP-induction (Supplementary Figure S5B), confirming membrane localization.

CAD1 has been identified in plasma membrane fractions in several independent proteomics studies (Alexandersson et al. 2004; De Michele et al. 2009; Elmore et al. 2012). To confirm the subcellular localization of CAD1, we transformed cad1-5 mutants with pCAD1:gCAD1-GFP and tested genetic complementation of several cad1-5 phenotypes in two independent homozygous lines. We found that expression of pCAD1:gCAD1-GFP complemented the cad1-5 growth phenotype (Figure 4A) and enhanced expression of PR1 (Figure 4B), suggesting that C-terminally tagged CAD1 is functional. Confocal microscopy suggested that CAD1-GFP localized to both the cytoplasm and the plasma membrane in cad1-5/pCAD1:gCAD1-GFP seedlings (Figure 4C). However, our micrographs were not always clear, probably due to the relatively low expression of native promoter-driven CAD1. Therefore, to confirm these results, we performed subcellular fractionation experiments using cad1-5/pCAD1:gCAD1-GFP plants to isolate soluble and microsomal fractions. Enrichment of plasma membrane in our microsomal preparations was confirmed by the clear presence of the membrane transporter H\(^+\)-ATPase in western blots, while enrichment of cytosolic proteins in the soluble fraction was confirmed by probing for cytosolic fructose-1,6-biphosphatase (CFBPase). CAD1-GFP was clearly observed in both the microsomal and soluble fractions (Figure 4D), indicating that pools of CAD1 are found in both the cytosol and plasma membrane.

During our studies, we also generated transgenic lines expressing N-terminally tagged 35S:GFP:CAD1 in the cad1-5 background. Of these, 18/22 T\(_1\) lines displayed severe necrosis
and died on soil, resembling cad1 null alleles. To test if N-terminally tagged CAD1 resulted in a dominant-negative variant, we transformed Col-0 with both 35S:GFP-cCAD1 and 35S:cCAD1. Indeed, we found that 32/40 T1 lines of the genotype Col-0/35S:GFP-cCAD1 displayed necrotic and small rosette size whereas the Col-0/35S:cCAD1 control lines looked phenotypically similar to wild-type (Supplementary Figure S6). These data suggest that the integrity of the N-terminus is important for CAD1 function, possibly by regulating its stability, localization, or binding partners. This seems to be in contrast to NSL1, which can be recombinantly tagged on either the N- (Fukunaga et al. 2017) or C-terminus (Supplementary Figure S5) and still maintain function. Notably, a multiple sequence alignment of Arabidopsis MACPF proteins indicates variability in N-terminal residues (Supplementary Figure S7), including an extended region of ~10 residues in CAD1.

Immunity is a complex trait involving many classes of proteins and layers of regulation. While proteins with MACPF motifs are known to function as pore-forming proteins in other organisms, their molecular function in plants remains unclear. Do these proteins form pores in membranes? And if so, how? Deploying pore-forming proteins as a defense mechanism would provide plants with a potent antimicrobial agent, and may therefore be targeted by pathogen effectors in the tug-of-war between host and microbe. This hypothesis could provide additional support to the suggestion that CAD1 and NSL1 are guarded by the immune system. Alternatively, these proteins may form pores in their own or neighboring cell membranes, which could release DAMPs and potentiate immune signaling. Perforin contains both a membrane-associating β-barrel region and membrane-inserting CC motifs, features that are essential for pore formation in mammalian MACPF domain proteins (Law et al. 2010). Neither CAD1 nor NSL1 were predicted to contain any transmembrane regions using TMHMM Server v. 2.0; however, as both CAD1 and NSL1 localize to the cell membrane it is feasible that they form a heteromeric complex which together create a pore, similar to the complement proteins. In this scenario, it may be the integrity of the protein complex that is important for cell viability and the regulation of defense pathways. While this is supported by the finding that both cad1 and nsl1 mutants display similar (non-redundant) phenotypes, well-controlled protein–protein association studies are required to strengthen and test this hypothesis.

Figure 4 Subcellular localization of CAD1. (A) Plants were photographed after growth in a short-day chamber 5 weeks post-germination. (B) Real time quantitative reverse-transcription PCR of PR1 was performed and plotted relative to expression of UBOX. Values are means ±/standard error from three independent experiments. (C) Confocal microscopy images of cad1-5/pCAD1:gCAD1-GFP transgenic lines #4 and #5 with both the green and red channels overlay; green indicates GFP fluorescence and red indicates auto-fluorescence from chloroplasts. (D) Western blot on soluble and microsomal protein fractions of cad1-5/pCAD1:gCAD1-GFP transgenic line #4. Detection of cytosolic CFBPase or membrane-bound H+ -ATPase demonstrates successful fractionation. CAD1-GFP is clearly detected in both the soluble and microsomal fractions, indicating dual-localization. All experiments were repeated at least three times with similar results. Statistically significant (P<0.05) groups were analyzed by ANOVA followed by Tukey’s post-hoc test and are indicated by lower-case letters.
We predict that uncovering the molecular function and biochemical mechanism of plant MACPF-containing proteins will be an exciting advance in plant cell biology.

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Author contributions

J.M. and C.Z. designed the study and funded the research; J.M., D.R.H., M.B., K.T., and S.A.P. performed experiments; I.S. and K.R.S. performed supporting experiments that are not shown. J.M. wrote the paper with input from all authors.

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References

Aarts N, Metz M, Holub E, Staskawicz B, Daniels MJ, et al. 1998. Different requirements for EDS1 and NDR1 by disease resistance genes define at least two R gene-mediated signaling pathways in Arabidopsis. Proc Natl AcadSci USA. 95:10306–10311.

Alexandersson E, Saalbach G, Larsson C, Kjellborn P. 2004. Arabidopsis plasma membrane proteomics identifies components of transport, signal transduction and membrane trafficking. Plant Cell Physiol. 45:1543–1556.

Asada Y, Yamamoto M, Tsutsui T, Yamaguchi J. 2011. The Arabidopsis NSL2 negatively controls systemic acquired resistance via hypersensitive response. Plant Biotechnology. 28:9–15.

Bayly-Jones C, Bubek D, Dunstone M A. 2017. The mystery behind membrane insertion: a review of the complement membrane attack complex. Phil Trans R Soc B. 372:20160221.

Bent A. 2006. Arabidopsis thaliana floral dip transformation method. Methods Mol Biol. 343:87–103.

Bredow M, Sementchoukov I, Siegel K, Monaghan J. 2019. Pattern-triggered oxidative burst and seedling growth inhibition assays in Arabidopsis thaliana. J Vis Exp. 147:e59437.

Bustin SA, Renes V, Garson JA, Hellemans J, Huggett J, et al. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 55:611–622.

Century KS, Shapiro AD, Repetti PP, Dahlbeck D, Holub E, et al. 1997. NDR1, a pathogen-induced component required for Arabidopsis disease resistance. Science 278:1963–1965.

Chen T, Nomura K, Wang X, Sohrabi R, Xu J, et al. 2020. A plant genetic network for preventing dysbiosis in the phyllosphere. Nature 580:653–657.

Coppinger P, Repetti PP, Day B, Dahlbeck D, Mehliert A, et al. 2004. Overexpression of the plasma membrane-localized NDR1 protein results in enhanced bacterial disease resistance in Arabidopsis thaliana. Plant J. 40:225–237.

Couto D, Zipfel C. 2016. Regulation of pattern recognition receptor signalling in plants. Nat Rev Immunol. 16:537–552.

Crooks GE, Hon G, Chandonia J-M, Brenner SE. 2004. WebLogo: a sequence logo generator. Genome Res. 14:1188–1190.

Dangl JL, Jones JDG. 2019. A pentagonal plant inflammasome. Science 364:31–32.

De Michele R, Vuuro E, Rigo C, Costa A, Elviri L, et al. 2009. Nitric oxide is involved in cadmium-induced programmed cell death in Arabidopsis suspension cultures. Plant Physiol. 150:217–228.

Dou D, Zhou J-M. 2012. Phytopathogen effectors subverting host immunity: different foes, similar battleground. Cell Host Microbe. 12:484–495.

Elmore JM, Liu J, Smith B, Phinney B, Coaker G. 2012. Quantitative proteomics reveals dynamic changes in the plasma membrane during Arabidopsis immune signaling. Mol Cell Proteomics. 11: M111.014555.

Emanuelsson O, Brunak S, von Heijne G, Nielsen H. 2007. Locating proteins in the cell using TargetP, SignalP and related tools. Nat Protoc. 2:959–971.

Etherington GJ, Monaghan J, Zipfel C, MacLean D. 2014. Mapping mutations in plant genomes with the user-friendly web application CandiSNP. Plant Methods. 10:41.

Feyjs BJ, Wiermer M, Bhat RA, Medina-Escobar N, et al. 2005. Arabidopsis SENSESCENCE-ASSOCIATED GENE1 stabilizes and signals within an ENHANCED DISEASE SUSCEPTIBILITY1 complex in plant innate immunity. Plant Cell. 17:2601–2613.

Fukunaga S, Sogame M, Hata M, Singkaravanit-Ogawa S, Pilailewskia-Bednarek M, et al. 2017. Dysfunction of Arabidopsis MACPF domain protein activates programmed cell death via tryptophan metabolism in MAMP-triggered immunity. Plant J. 89:381–393.

Gimenez-Ibanez S, Hann DR, Ntoukakis V, Petutschnig E, Lipka V, et al. 2009. AvrPtoB targets the LysM receptor kinase CERK1 to promote bacterial virulence on plants. Curr Biol. 19:423–429.

van der Hoorn RAL, Kamoun S. 2008. From guard to decoy: a new model for perception of plant pathogen effectors. Plant Cell. 20: 2009–2017.

Jones JDG, Dangl JL. 2006. The plant immune system. Nature 444:2430–2455.

Lapin D, Kovacova V, Sun X, Dongus JA, Bhandari D, et al. 2019. A coevolved EDS1-SAG101-NRG1 module mediates cell death signaling by TIR-domain immune receptors. Plant Cell. 31: 2430–2455.
Lapin D, Bhandari DD, Parker JE. 2020. Origins and immunity networking functions of EDS1 family proteins. Annu Rev Phytopathol. 58:253–276.

Law R H P, Lukoyanova N, Voskoboinik I, Caradoc-Davies T T, Baran K, et al. 2010. The structural basis for membrane binding and pore formation by lymphocyte perforin. Nature. 468:447–451.

Ma S, Lapin D, Liu L, Sun Y, Song W, et al. 2020. Direct pathogen-induced assembly of an NLR immune receptor complex to form a holoenzyme. Science 370:eabe3069.

Macho AP, Zipfel C. 2014. Plant PRRs and the activation of innate immune signaling. Mol. Cell. 54:263–272.

Martin R, Qi T, Zhang H, Liu F, King M, et al. 2020. Structure of the activated ROQ1 resistosome directly recognizing the pathogen effector XopQ. Science 370:eabd9993.

McCann HC, Nahal H, Thakur S, Guttman DS. 2012. Identification of innate immunity elicitors using molecular signatures of natural selection. Proc Natl Acad Sci USA. 109:4215–4220.

McHale L, Tan X, Koehl P, Michelmore RW. 2006. Plant NBS-LRR proteins: adaptable guardians. Genome Biol. 7:212.

Monaghan J, Xu F, Gao M, Zhao Q, Palma K, et al. 2009. Two Prp19-like U-box proteins in the MOS4-associated complex play redundant roles in plant innate immunity. PLoS Pathog. 5:e1000526.

Monaghan J, Matschi S, Shorinola O, Rovenich H, Matei A, et al. 2014. The calcium-dependent protein kinase CPK28 buffers plant immunity and regulates BIK1 turnover. Cell Host Microbe. 16:605–615.

Morita-Yamamuro C, Tsutsui T, Sato M, Yoshioka H, Tamaoki M, et al. 2005. The Arabidopsis gene CAD1 controls programmed cell death in the plant immune system and encodes a protein containing a MACPF domain. Plant Cell Physiol. 46:902–912.

Nakagawa T, Kurose T, Hino T, Tanaka K, Kawamukai M, et al. 2007. Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. J Biosci Bioeng. 104:34–41.

Ni T, Gilbert RJC. 2017. Repurposing a pore: highly conserved perforin-like proteins with alternative mechanisms. Philos Trans R Soc Lond B Biol Sci. 372:20160212.

Noutoshi Y, Kuromori T, Wada T, Hirayama T, Kamiya A, et al. 2006. Loss of Necrotic Spotted Lesions 1 associates with cell death and defense responses in Arabidopsis thaliana. Plant Mol Biol. 62:29–42.

Rodríguez E, Ghoul HE, Mundy J, Petersen M. 2016. Making sense of plant autoimmunity and “negative regulators”. Febs J. 283:1385–1391.

Rosado C J, Buckle AM, Law RHP, Butcher RE, Kan W-T, et al. 2007. A common fold mediates vertebrate defense and bacterial attack. Science 317:1548–1551.

Roux M, Schwessinger B, Albrecht C, Chinchilla D, Jones A, et al. 2011. The Arabidopsis leucine-rich repeat receptor–like kinases BAK1/SERK3 and BKK1/SERK4 are required for innate immunity to hemibiotrophic and biotrophic pathogens. Plant Cell. 23:2440–2455.

Schulze B, Mentzel T, Jehle AK, Mueller K, Beeler S, et al. 2010. Rapid heteromerization and phosphorylation of ligand-activated plant transmembrane receptors and their associated kinase BAK1. J Biol Chem. 285:9444–9451.

Schwessinger B, Roux M, Kadota Y, Ntoukakis V, Sklenar J, et al. 2011. Phosphorylation-dependent differential regulation of plant growth, cell death, and innate immunity by the regulatory receptor-like kinase BAK1. PLoS Genet. 7:e1002046.

Segonzac C, Monaghan J. 2019. Modulation of plant innate immune signaling by small peptides. Curr Opin Plant Biol. 51:22–28.

Stegmann M, Monaghan J, Smakowska-Luzan E, Rovenich H, Lehner A, et al. 2017. The receptor kinase FER is a RALF-regulated scaffold controlling plant immune signaling. Science 355:287–289.

Takahashi D, Nakayama T, Miki Y, Kawamura Y, Uemura M. 2014. Proteomic approaches to identify cold-regulated plasma membrane proteins. Methods Mol Biol. 1166:159–170.

Tian L, Li X. 2020. Enzyme formation by immune receptors. Science 370:1163–1164.

Tsutsui T, Morita-Yamamuro C, Asada Y, Minami E, Shibuya N, et al. 2006. Salicylic acid and a chitin elicitor both control expression of the CAD1 gene involved in the plant immunity of Arabidopsis. Biosci Biotechnol Biochem. 70:2042–2048.

Tsutsui T, Asada Y, Tamaoki M, Ikeda A, Yamaguchi J. 2008. Arabidopsis CAD1 negatively controls plant immunity mediated by both salicylic acid-dependent and -independent signaling pathways. Plant Sci. 175:604–611.

Wang J, Hu M, Wang J, Qi J, Han Z, et al. 2019a. Reconstitution and structure of a plant NLR resistosome conferring immunity. Science 364:eaav5870.

Wang J, Wang J, Hu M, Wu S, Qi J, et al. 2019b. Ligand-triggered allosteric ADP release primes a plant NLR complex. Science 364:eaav5868.

Wildermuth MC, Dewdney J, Wu G, Ausubel FM. 2001. Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature 414:562–565.

Yu L, Liu D, Chen S, Dai Y, Guo W, et al. 2020. Evolution and expression of the membrane attack complex and perforin gene family in the Poaceae. IJMS 21:https://doi.org/10.3390/ijms21165736.

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