The involvement of Arabidopsis calmodulin in plant immunity against Pseudomonas syringae

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The involvement of *Arabidopsis* calmodulin in plant immunity against *Pseudomonas syringae*

An Undergraduate Honors Thesis
Submitted in Partial fulfillments of
University Honors Program Requirements
University of Nebraska-Lincoln

by
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March 13, 2017

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Abstract

Calmodulin (CaM) is a calcium-binding protein present in all eukaryotic cells and is essential for many cellular functions. Calcium-specific binding to CaM induces a conformational change in CaM that affects its interaction with different target proteins, influencing the activity of proteins that bind it. Through calcium signal transduction, CaM mediates a variety of important cellular processes, including plant immunity. The *Arabidopsis* genome encodes 7 CaM genes that are highly conserved and homologous. While the involvement of CaMs in several eukaryotic processes is known, many of their biological functions in *Arabidopsis* remain largely unknown. *Pseudomonas syringae* pv. *tomato* DC3000 is pathogenic on tomato and *Arabidopsis*. It uses a type III secretion system to inject effector proteins into plant cells to favor pathogenicity. A major role of these type III effectors is to suppress plant immunity. It was recently found that one *P. syringae* effector (HopE1) utilizes CaM as a co-factor inside plant cells to target MAP65-1 and dissociate it from the microtubule network, thus inhibiting the secretion of important immune-related proteins. The main goal of this investigation was to examine *Arabidopsis* CaMs in order to identify those that may play a larger role in plant immunity to *P. syringae*. *Arabidopsis* mutated for each of the seven CAM genes were screened with a series of immunity assays and the expression levels of the CAM genes in wild type *Arabidopsis* in response to immunity activators were measured. Here, it was shown that cam4 and cam5 mutants, and cam1cam4 and cam2cam5 double mutants, were more resistant to infection by *P. syringae* (Figure 4). Additionally, it was shown that the expression levels of CAM1, CAM2, CAM3, and CAM5 were induced in wild type *Arabidopsis* upon activation of PTI and ETI (Figure 6-Figure 8).
Acknowledgements

Thanks to my mentor in the lab, PhD student Panya Kim, and to Dr. Jim Alfano, the members of the Alfano Lab, my thesis advisors Dr. Dave Gosselin and Dr. Wayne Riekhof, and thesis reader, Christine Haney, the UNL Honors Program, and to the UCARE program for their support of my educational and research endeavors.
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1. Introduction

1.1 Study Description

As world population continues to grow, greater stress is placed on our world’s resources. Society will also experience a more unpredictable environment because of global climate change. Both of these factors will place stress on the food supply. It will be vitally important to have plants that can withstand these to meet the demand for higher food production. 1. *Pseudomonas syringae* (*P. syringae*) is a prominent crop pathogen in the US and the world. It has the unique ability to cause disease in at least 180 plant species (Valencia-Botín and Cisneros-López, 2011). 2. International crop yield losses due to this pathogen are estimated to range from 5 to 50% (Valencia-Botín and Cisneros-López, 2011). Calmodulin (*CaM*) is a highly-conserved calcium-binding protein present in all eukaryotic cells. Through calcium signal transduction, *CaM* mediates a variety of important processes within plant cells, including the immune response. However, there is little currently known about the role that calmodulin plays in the immune response of the model plant, *Arabidopsis*.

*P. syringae* pathovar *tomato* DC3000 infects tomato and the model plant, *Arabidopsis*. *Arabidopsis* demonstrates infection patterns similar to important crop plants which the pathogen also infects and can be used to study the role of calmodulin in plant immunity. The genomes of both the pathogen and the model plant are known, allowing the investigation of host-microbe interactions at the genomic level. These characteristics, along with the relatively short generation time of *Arabidopsis*, make it an ideal model for in-situ experimentation when there are time and space constraints, as well as the need for multiple generations of plants and replication of experiments.
1.2 Pseudomonas syringae Background

*P. syringae* is a Gram-negative extracellular pathogen that lives in the plant tissue between plant cells. *P. syringae* can infect virtually all plants, but many strains of the bacterium are host-specific and therefore the species has been separated into more than 50 different pathovars based on the host that each infects (Gardan et al., 1999). One of these pathovars, *P. syringae* pv. *tomato* DC3000, is pathogenic on tomato plants and on the model plant *Arabidopsis*. In susceptible hosts, this pathogen multiplies and spreads throughout the plant, where it causes chloric and necrotic lesions. In resistant hosts, the pathogen elicits hypersensitive response (HR), which is a plant defense response that ultimately prevents the spread of the microbial pathogen infection by eliciting rapid death of plant cells in the local region surrounding an infection (Coll et al., 2011). The type III protein secretion system is an important feature of *P. syringae* that allows it to grow in plants and cause disease by injecting bacterial proteins, called type III effectors, into host plant cells similar to the way a needle and syringe work (Figure 1) (Guo et al., 2009). DC3000 has 36 type III effectors that are known to suppress plant immunity through a number of different mechanisms within the plant cell (Block and Alfano, 2011).

The plant has a number of ways in which it is able to detect the presence of *P. syringae*, and other pathogens, in and around its cells. The innate immune system of *Arabidopsis* and other plants detects the presence of pathogens using two types of immune receptors. The first involves the recognition of pathogen-associated molecular patterns (PAMPs, also known as MAMPs, microbe-associated molecular patterns). PAMPs are molecules or molecular patterns that are highly conserved across microorganisms. Receptors that recognize these patterns are located on the outside of the plant cell surface (Jones and Dangl, 2006). This recognition event induces an
immune response in the plant called PAMP-triggered immunity (PTI). The second way the plant
detects pathogenic microorganisms is with internal immune receptors that recognize specific
pathogen effector proteins and induce an immune response called effector-triggered immunity
(ETI) (Jones and Dangl, 2006). Resistant plants can recognize specific bacterial type III effectors
using intracellular nucleotide-binding sites, which are leucine-rich repeat immune receptors
called resistance I proteins (Jones and Dangl, 2001). This recognition is achieved by R-proteins
that monitor specific plant proteins for modification by type III effectors and then respond by
activating ETI. Both PTI and ETI activate similar signaling pathways and immune responses;
however, ETI generally activates them in a more prolonged, vigorous fashion than PTI, and
usually includes the HR, a programmed cell death response mentioned previously.

Figure 1. A depiction of the mechanism of the *P. syringae* (*Pst. DC3000*) type III secretion system
(orange oval) as well as the known effector targets within the host plant cell. (Note: other targets have
since been identified. For example, cited in this paper are two articles proposing new targets of effectors.
The first is that a target of HopD1 is the *Arabidopsis* transcription factor, NTL9 (Block et al., 2013) and
the other identifies a target of HopE1, which is calmodulin (Guo et al., 2016)) (Figure from Xin and He,
2013).
1.3 Calmodulin Background

Calmodulin (CaM) is a calcium-binding protein present in all eukaryotic cells. It is essential for many cellular functions. Calcium-specific binding to CaM induces a conformational change in CaM that affects its interaction with different target proteins and influences the activity of proteins that bind it (Yang and Poovaiah, 2003). It is known that the Ca\(^{2+}\) cation fluxes across the plant plasma membrane into the cytosol upon initial recognition of a pathogen (Lecourieux et al., 2006). Through calcium signal transduction, CaM mediates a variety of important cellular processes, including the immune responses (Cheval et al., 2013). The Arabidopsis genome encodes seven genes for CaM (CAM genes) that are highly homologous and relatively uniformly expressed (McCormack et al., 2005). These seven genes occur in the following four isoforms; CaM1/4, CaM2/3/5, CaM6, and CaM7, differing from one another by just one to five amino acid residues (Poovaiah et al., 2013). The biological functions of the Arabidopsis CaMs remain largely unknown.

As mentioned previously, P. syringae pv. tomato DC3000 is pathogenic on tomato plants and Arabidopsis. It uses a type III secretion system to inject type III effector proteins into plant cells to favor pathogenicity. A major role of type III effectors is the suppression of plant immunity. It has recently been found that one P. syringae effector, called HopE1, uses the host CaM as a co-factor to target the microtubule-associated protein MAP65-1 (Figure 2) (Guo et al, 2016). In fact, CaM only interacted with MAP65-1 in the presence of HopE1 (Guo et al, 2016). MAP65-1 is a vital component of the cell’s microtubule network and plays an important role in shuttling immune-related proteins out of the cell where they can fight the pathogen in what is known as cell wall-based extracellular immunity (Guo et al, 2016).
Figure 2. Left: depiction of a *P. syringae* *hopE1* mutant that lacks the effector HopE1 and the resultant null effect on MAP65-1 association with the microtubule network. Right: illustration of *P. syringae* expressing HopE1 and the action of the effector after it is injected into the host cell, binding calmodulin and dissociating MAP65-1 from the microtubule network, resulting in suppression of cell wall-based immunity (Guo et al., 2016).

1.4 Statement of Purpose

*Arabidopsis CAM* genes are investigated with the goal of identifying which ones play roles in plant immunity to *P. syringae*. The purpose of this paper is to improve the understanding of the action of calmodulin within the host cell. Understanding the extent to which CaM influences the plant’s resistance to pathogens in the field will, in turn, aid in the effort to increase plant health and thus crop yield. This research aims to add a small, but significant piece to this much larger puzzle through investigating the role that calmodulin plays in *Arabidopsis* plant immunity to *P. syringae*. This investigation is expected to identify new components of plant immunity related to the role of the calcium-sensing protein, calmodulin.
2. Methods

2.1 T-DNA Mutant Genotyping

T-DNA mutants in four of the seven CAM genes in Arabidopsis were obtained from a company called the Salk Institute and denoted CAM1, CAM2, CAM4, and CAM5. T-DNA mutants contain foreign DNA that have been inserted into a gene of interest as a means of disrupting gene function. This involves the use of transfer DNA or T-DNA in the Arabidopsis genome. To check that the foreign DNA was inserted into the correct location in the Arabidopsis genome (within the correct CAM gene), DNA from the mutants was extracted and the polymerase chain reaction (PCR) method was used. PCR with gene specific (GS) primers and/or T-DNA border (TB) primers (Table 1) allowed for the identification of individual plants that were homozygous for a particular T-DNA mutation. Double mutants were created by crossing successful single mutants in two different CAM genes and then screening for homozygosity in the double mutation in later generations by the PCR method.

<table>
<thead>
<tr>
<th>Calmodulin T-DNA mutants</th>
<th>Gene specific primers</th>
<th>Product size</th>
</tr>
</thead>
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<tr>
<td>CAM5 (AT2G27030) SALK_007371.56.00</td>
<td>SALK_007371-LP GGTGTTGGAGATTCTCATTCATC</td>
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</tr>
<tr>
<td>CAM5 (AT2G27030) SALK_007371-RP AAGAAGCTTTCAGGTTTTCG</td>
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</tr>
<tr>
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<tr>
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<td></td>
</tr>
<tr>
<td>CAM4 (AT1G66410) SALK_149142.30.45</td>
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</tbody>
</table>

Table 1. Calmodulin T-DNA mutants and primer list, including gene specific (GS) primers and a T-DNA insert border primer (TB) and PCR product sizes. LP- left primer, RP- right primer. SALK_# given by Salk Institute, the company that created the mutants.
2.2 Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) of Arabidopsis CAM T-DNA mutants was done to ensure that mutants appropriately lacked expression of the associated CaM proteins. Total RNA was extracted from three-week-old T-DNA mutant lines. Each CAM gene expression was evaluated using qRT-PCR ($P < 0.05$). Transcript levels of the CAM genes in the mutants were analyzed for efficiency of the transfer DNA insert and strength of the mutation. CAM gene expression in Col-0, the wild type Arabidopsis, was used as a control.

2.3 Pathogenicity Assay

Pathogenicity assays in Arabidopsis were done to screen the mutants for alteration in pathogenesis (Block et al., 2014). P. syringae DC3000 and hrcC, a mutant strain unable to cause infection (used as a control), were separately grown on solid LM media with rifampicin, inoculated into 1mM MgCl$_2$, and adjust to a cell suspension of OD$_{600}=0.2$ ($2 \times 10^8$ cells/ml). Arabidopsis T-DNA mutants and Col-0 were spray inoculated with these suspensions. Tissue samples were taken immediately after drying (Day 0) and three days post inoculation (Day 3). Four leaf discs were taken from each plant to adjust for minor variations between leaves. Samples were then ground and plated in dilutions. Bacterial growth numbers were counted three days from when Day 0 and Day 3 samples were taken and the data was plotted for analysis. This assay in Arabidopsis can be performed to discover susceptible or resistant phenotypes based on the bacterial growth number and disease symptoms. Resistant phenotypes are those that show less bacterial growth or disease symptoms compared to the wild type Col-0. A susceptible phenotype is one that shows equal to or greater bacterial growth or disease symptoms than Col-0. In the diseased phenotype, the leaves of Arabidopsis will turn yellow and necrotic. Results are averaged after doing this assay in triplicate.
2.4 ROS Production Assay

*Arabidopsis* CaM mutants and wild type (Col-0) as a control were induced by flg22 and the ROS production assay was conducted following the protocol previously described (Asai and Yoshioka, 2008). Leaf disks were taken from 4-week-old plants, placed in water and incubated overnight. The following day, they were placed in 0.5mM L-012 in 10mM MOPS/KOH buffer treated with 1µl flg22. ROS intensity was measured by counting photons from L-012-mediated luminescence monitored with a Synergy 5 luminometer (BioTek).

2.5 Callose Deposition Assay

Callose production assays were done following the previously described protocol (Nguyen et al., 2010). Leaves of 6-week-old *Arabidopsis* single cam mutants and Col-0 as a control were syringe infiltrated with 2 µM flg22 and harvested 16 h later, cleared with 100% ethanol at 37°C overnight and washed twice with 70% ethanol and three times with water. Completely cleared leaves were stained with 0.1% alanine blue in a solution of 150mM K2HPO4, pH 9.5, and incubated in the dark for 30 minutes. Stained leaves were mounted on slides with 60% glycerol and observed by epifluorescence microscopy using UV light and the callose deposits were detected by alanine blue fluorescence and counted. Quantification of callose deposits were made by using the ImageJ software.

2.6 CAM Gene Induction Assay

Three-week-old wild type *Arabidopsis* (Col-0) expressing all seven calmodulins were syringe-infiltrated with either 1 µM flg22, 1 mM SA, or DC3000(pavrRpm1), along with mock (ddiH₂O) as a control. Total RNA was extracted 18 h and 24 h after inoculation, respectively. The transcript levels of CAM1, CAM2, CAM3, CAM4, CAM5, CAM6, and CAM7, as well as PRI (pathogenesis-related protein 1) or FRK1 (FLG22-induced receptor-like kinase 1) as controls,
were determined by qRT-PCR. The expression of CAMs and PRI was normalized to the expression of ACT2. The data are shown as the mean ± s.e.m. from three independent biological replicates and a significant difference with P<0.01 was analyzed with one-way ANOVA analysis.

3. Results

3.1 T-DNA Mutant Genotyping

The expression levels of each CAM gene in the mutants were measured by qRT-PCR and compare to wild type Arabidopsis (Col-0). The duplicate salk numbers associated with each CAM gene represent duplicate mutant lines and have different gene expression levels of the gene they are mutated for. All CAM T-DNA mutants received from the Salk Institute had significantly decreased expression of their associated CAM genes. A 70-80% decrease in gene expression of CAM1 and CAM2 was observed. Nearly 100% of CAM5 gene expression was eliminated for all lines tested. CAM4 gene expression had the lowest reduction in the CAM4 T-DNA mutants, resulting in 40-60% of wild type Col-0 expression of CAM4 (Figure 3).

![Figure 3. Isolation of CAM T-DNA mutants. (A-D) Transcript levels of CAM1, CAM2, CAM4, and CAM5 in wild type Arabidopsis (Col-0) and in cam1, cam2, cam4, and cam5 T-DNA mutants. Total RNA was extracted from three-week-old plants. Each CAM gene expression was evaluated using qRT-PCR (P < 0.05).](image-url)
3.2 Pathogenicity Assay

Pathogenicity assays were conducted on the previous four cam mutants (cam1, cam2, cam4, and cam5) and two double mutants, cam1cam4 and cam2cam5. Through multiple trials and statistical analysis, it was determined that cam4 and cam5 mutants and cam1cam4 and cam2cam5 double mutants have increased resistance to P. syringae. A reduction in bacterial growth of 0.25 cfu/cm² was seen for the single mutants (b), 0.5 cfu/cm² reduction was observed for cam2cam5 (c), and 1.0 cfu/cm² for cam1cam4 (c) at Day 3 compared to Col-0 (Figure 4). The two double mutants had statistically significantly less bacterial growth (P<0.05) than the single more resistant strains, cam4 and cam5, following Day 3, as seen by the letters b and c, representing statistical significance between the groups.

Figure 4. cam4 and cam5 mutants and cam1cam4 and cam2cam5 double mutants have increased resistance to P. syringae. DC3000 infection assays were done using wild type (Col-0), cam1, cam2, cam4, cam5, cam1cam4, and cam2cam5 mutants. Four-week-old plants were spray-inoculated with a P. syringae pv. tomato DC3000 suspension (OD₆₀₀=108 cells/ml). Bacterial growth in leaves was determined 0 and 3 days after inoculation (P<0.05).
3.3 ROS and Callose Assays

Reactive oxygen species (ROS) and callose assays were conducted on the four cam mutants and Col-0. cam1 and cam4 mutants had reduced levels of flg22-induced ROS production, 1.5x10^4 relative luminescence unit (RLU) less than wild type. cam1, cam2, and cam4 exhibited reduced levels of flg22-induced callose deposition. cam1 had 400 less, cam2 had 325 less, and cam4 had 350 less callose deposits than wild type (Figure 5). Values are mean ± SEM. (n=20).

![Figure 5](image.png)

Figure 5. (A) Arabidopsis cam1 and cam4 mutants have reduced levels of flg22-induced ROS production. RLU, relative luminescence unit. (B) cam1, cam2, and cam4 exhibit reduced levels of flg22-induced callose deposition. Quantification of callose deposits using the ImageJ software. Values are mean ± SEM. (n=20).

3.4 CAM Gene Induction by SA

The relative gene expression levels of all seven CAM genes upon induction by salicylic acid (SA), a defense hormone that controls plant defense against pathogens (Nimchuk et al., 2003), were measured in wild type Arabidopsis. CAM1, CAM3, and CAM5 genes were induced by SA (Figure 6). The expression level of CAM1 after treatment with SA increased 8 times compared to that of mock treatment, CAM3 expression increased 5 times after SA compared to
mock, and \textit{CAM5} increased 7 times compared to its expression level after mock treatment.

\textit{CAM2}, \textit{CAM4}, \textit{CAM6}, and \textit{CAM7} were not significantly induced by treatment with SA (P<0.05).

\textbf{Figure 6.} \textit{CAM1}, \textit{CAM3}, and \textit{CAM5} genes are induced by SA. (A-B) Three-week-old \textit{Arabidopsis} were infiltrated with mock or 1mM SA. Total RNA was extracted 24 h after inoculation. (A) The transcript levels of \textit{CAM1}, \textit{CAM2}, \textit{CAM3}, \textit{CAM4}, \textit{CAM5}, \textit{CAM6}, and \textit{CAM7} were determined with qRT-PCR (P < 0.05). Values are mean ± s.e. (n=3). (B) \textit{PR1} transcript levels were used as an experimental control and determined with qRT-PCR (P < 0.05). Values are mean ± s.e. (n=3).

\textbf{3.5 CAM Gene Induction by flg22}

\textit{CAM1}, \textit{CAM2}, and \textit{CAM3} genes were induced by flg22, a bacterial flagellin protein known to trigger PTI. \textit{CAM1} gene expression was elevated by flg22 to levels 4 times that of mock, \textit{CAM2} expression was elevated to levels 5 times that of mock, and \textit{CAM3} expression levels increased by 3 times the level of gene expression following mock treatment. \textit{CAM4}, \textit{CAM5}, \textit{CAM6}, and \textit{CAM7} genes were not significantly induced by flg22 (P<0.01) (Figure 7).
Figure 7. CAM1, CAM2, and CAM3 genes are induced by 1 µM flg22. Three-week-old wild type were infiltrated with mock or 1mM flg22. Total RNA was extracted 18 h after inoculation. (A) The transcript levels of CAM1, CAM2, CAM3, CAM4, CAM5, CAM6, CAM7, and FRK1 were determined with qRT-PCR (P < 0.01). Values are mean ± s.e. (n=3). (B) The transcript levels of FRK1 was used as an experimental control.

3.6 CAM Gene Induction by DC3000(pavrRpm1)

CAM2 and CAM5 genes were induced by P. syringae pv. tomato DC3000(pavrRpm1), a type III effector known to trigger ETI. After induction with DC3000(pavrRpm1), CAM2 gene expression levels increased to 7 times that of mock treatment and CAM5 expression levels increased to 4 times that of mock (Figure 8). CAM1, CAM3, CAM4, CAM6, and CAM7 were not significantly induced by DC3000(pavrRpm1) (P<0.01).

Figure 8. CAM2 and CAM5 genes are induced by P. syringae pv. tomato DC3000(pavrRpm1). Three-week-old wild type plants were infiltrated with mock or DC3000(pavrRpm1). Total RNA was extracted 24 h after inoculation. The transcript levels of CAM1, CAM2, CAM3, CAM4, CAM5, CAM6, CAM7, and PRI were determined with qRT-PCR (P < 0.01). Values are mean ± s.e. (n=3). The transcript level of PRI was used as a control.
4. Discussion

The purpose of this study was to examine the role of calmodulin in Arabidopsis immunity to *P. syringae*. These data support the hypothesis that certain calmodulin play a larger role in the immune response, and these were found to be CAM1, CAM2, CAM3, and CAM5 genes, which were upregulated upon induction by SA, flg22, and DC3000(*pavrRpm1*) (Figures 6-8). These results make sense in the context of previous studies as CaM has been shown to mediate the plant immune response (Cheval et al., 2013). Furthermore, *cam4, cam5, cam1cam4*, and *cam2cam5* mutants resulted in increased immunity to *P. syringae* (Figure 4). Additionally, all of the mutant lines *cam1, cam2, cam4*, and *cam5*, either decreased ROS, callose production, or both (Figure 5). At first glance, it seems contrary that knocking out genes encoding for calmodulin would increase the plant’s resistance to *P. syringae*, while the very same genes are upregulated when the plant is exposed to triggers of immunity (SA, flg22, and DC3000(*pavrRpm1*)).

However, previous studies reveal that CaM is involved in both positive and negative immune response pathways, an example lies in CaM-binding transcription factors, which have been shown to both positively and negatively regulate gene transcription (Cheval et al., 2013). It is very likely that these data illustrate both positive and negative roles of calmodulin on the plant immune response in yet clearly defined manners. It can be projected from these data that *CAM6* and *CAM7* genes play less significant roles in the plant immune response because they were not induced by SA, flg22, or DC3000(*pavrRpm1*) (Figures 6-8).

It was found that *cam1cam4* and *cam2cam5* double mutants have increased resistance to infection by *P. syringae* greater than that of *cam4* and *cam5* single mutants (Figure 4). This suggests that these *CAM* genes are not redundant and may function synergistically. This is supported by previous findings that downregulation of CaM gene expression, or loss-of-function
of CaM genes, have a strong effect on the plant immune response (Cheval et al., 2013). If the genes for calmodulin were redundant, one would have expected the single mutants to yield the same results as the double mutants.

As previously mentioned, CAM1, CAM2, CAM3, and CAM5 genes are induced in the wild type Arabidopsis by exposure to the triggers to the plant immune response used in this study. It is possible that the inductions of these genes in the wild type plant make the plant more susceptible to infection by P. syringae, as the effector HopE1 targets calmodulin and uses it as a co-factor to dissociate MAP65-1 from the microtubule network (Guo et al., 2016). Thus, the increase in abundance of calmodulin also increases the availability of that co-factor to HopE1. As a corollary to the previous hypothesis, the reduction of bacterial growth on cam4 and cam5 and the double mutants could be due to the inability of HopE1 to target as many calmodulin proteins, as these mutants are not expressing as much calmodulin as the wild type plant. With less calmodulin present for HopE1 to use as a co-factor, it would also not be able to dissociate MAP65-1 from the microtubule network as efficiently in one of these mutants as it would be able to do in the wild type plant. Further experimentation is needed to investigate these hypotheses and will be discussed later.

A third hypothesis is that the reduction of ROS and callose deposition by the cam1 and cam4, and cam1, cam2, and cam4 mutants respectively, could be due to decreased levels of calcium-signaling through calmodulin. It is known that these immune responses (ROS and callose formation) are triggered by calcium signaling mediated by calmodulin (Cheval et al., 2013). Therefore, mutants expressing lower levels of calmodulin would likely have decreased calcium signaling capabilities and thus less ROS and callose deposition.
Lastly, it is important to note that mutations in certain \( \text{CAM} \) genes have been shown to have detrimental effects on germination and heat tolerance, showing that calmodulin plays a number of important roles in the plant life cycle besides its role in plant immunity (Poovaiah et al., 2003). Knocking out calmodulin genes in plants should have a detrimental effect on plant health overall. Still, a larger benefit could be gained by a plant with mutations in calmodulin upon infection by \( P. \text{syringae} \) because the mutations allow the plant to avoid the actions of HopE1, and potentially other effectors, within the cell. That is, calmodulin is eliminated as a co-factor for HopE1 to dissociate the microtubule network in these mutants. Further research is required to elucidate the actions of HopE1 in CaM mutants.

5. Conclusion

5.1 Overview

Calmodulin mutants of \textit{Arabidopsis} were analyzed for their ability to resist pathogenesis by \( P. \text{syringae} \) in attempt to identify a single or a few \( \text{CAM} \) genes that play the most significant role(s) in plant immunity. Knowledge gathered from these experiments contributes to the larger body of knowledge regarding the action of \( P. \text{syringae} \) type III effectors within the plant cell, as well as the role of calmodulin in plant immunity against \( P. \text{syringae} \). By looking at the mutants’ response upon infection by \( P. \text{syringae} \), along with the gene expression of the seven \( \text{CAM} \) genes in wild type \textit{Arabidopsis} upon infiltration with triggers to the plant immune response, this research identified that mutations in specific calmodulin proteins reduced the pathogen growth on \textit{Arabidopsis} and that certain genes are upregulated upon induction of the immune response.

This research identified that \textit{cam4} and \textit{cam5} mutants and \textit{cam1cam4} and \textit{cam2cam5} double mutants have increased resistance to infection by \( P. \text{syringae} \) \textit{pv. tomato} DC3000 (Figure 4). \textit{cam1} and \textit{cam4} mutants suppress flg22-induced ROS and, \textit{cam1}, \textit{cam2}, and \textit{cam4} exhibit
reduced levels of flg22-induced callose deposition (Figure 5). CAM1, CAM3, and CAM5 genes were induced by SA (Figure 6). CAM1, CAM2, and CAM3 genes were induced by flg22 (Figure 7). CAM2 and CAM5 genes were induced by DC3000(pavrRpm1) (Figure 8).

5.2 Recommendations for Further Study

To improve this research, it would be beneficial to conduct the same experiments on all seven CaM mutants, as cam3, cam6, and cam7 were left out in the study due to the inability to access these mutant lines. A more comprehensive analysis is needed here in order to better address the research question. It would also be beneficial to test more double mutant lines to look for synergistic effects of CAM genes or to identify redundancy within the CAM genes among the isoforms. The future direction of this research could include conducting yeast-two-hybrid (Y2H) screening with the cam mutants, using CaM as the bait and HopE1 as the prey to test for interaction between CaM and HopE1 in the mutants. Another experiment that could be done is an in-vitro pull-down assay using CaM affinity resin (as was done in Guo et al. 2016 to identify HopE1 interaction with CaM) on each of the seven cam mutants with HopE1 to determine if a mutation in a specific CAM gene excludes the results found by Guo et al. 2016. This would address the following question; In the absence of specific CAM genes, is HopE1 disabled from dissociating MAP65-1 from the microtubule network? Further investigations are needed in order to continue to gain a better understanding of calcium-mediated signaling through calmodulin and the role of the protein in plant immunity against P. syringae.
References


