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Host Pathogen Interactions: Is Arabidopsis thaliana remembered by its Nemesis Pseudomonas syringae?

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Host-Pathogen Interactions: Is *Arabidopis thaliana* remembered by its Nemesis *Pseudomonas syringae?*

Plants, like animals, have an immune system that protects them from and deters invading pathogens during the infection process. Most people visualize only what is called the adaptive immune response of vertebrates when thinking of the immune system. The adaptive immune system is comprised of T cells, plasma B cells, and other forms of leukocytes that patrol the circulatory and lymphatic systems in addition to various tissues in vertebrates. These cells mediate defense responses

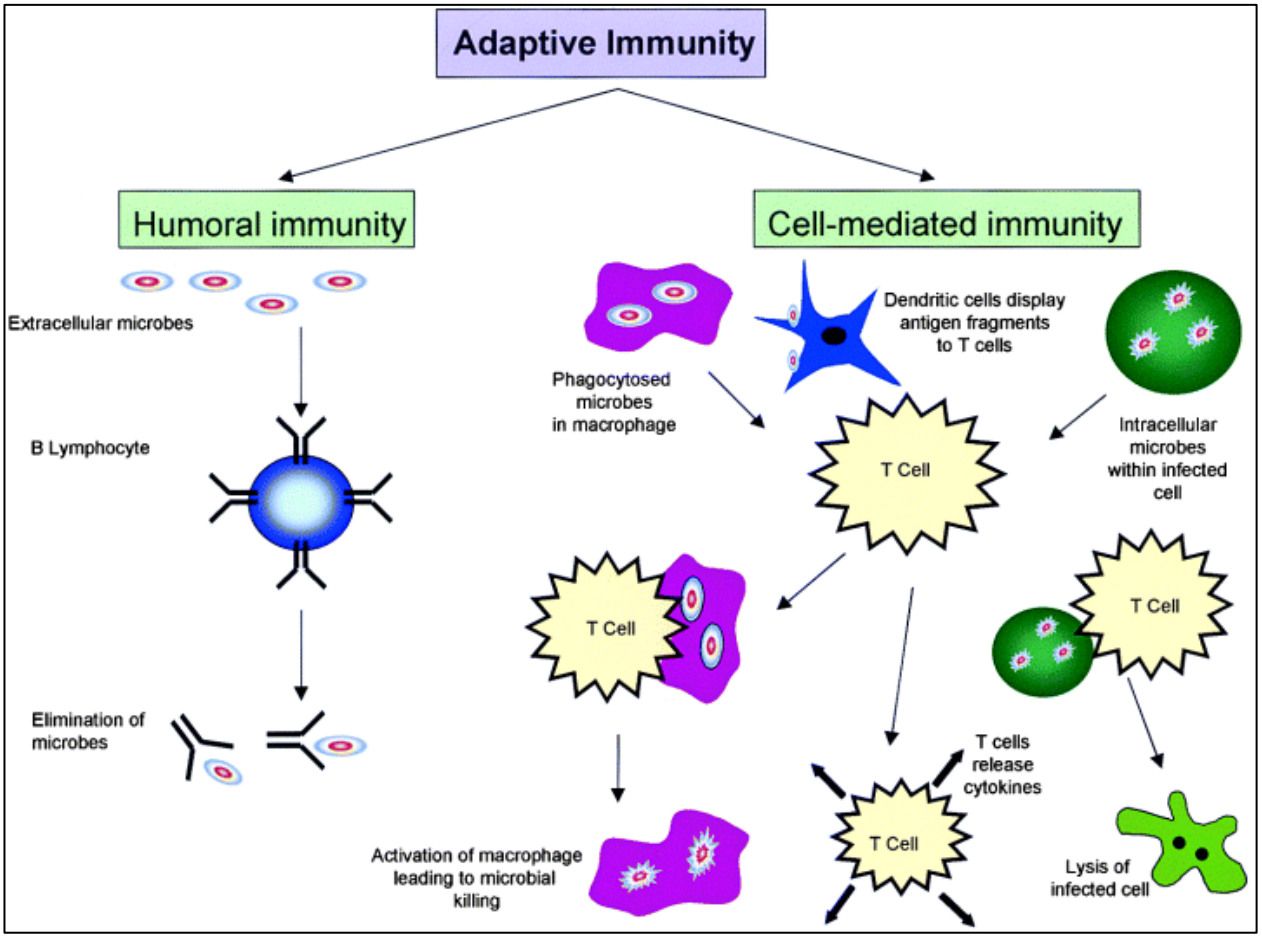

Figure 1 A simplified schematic of the adaptive immune system in mammals. There are two branches to the adaptive immune system: the humoral immunity and cell-mediated immunity. Humoral immunity consists of plasma B cells that secrete antibodies. Cell-mediated consist of helper T-cells and cytotoxic T-cells, which respectively activate macrophages and kill infected or tumorgenic cells. T-cells are activated by antigen –presenting cells like the dendritic cell pictured here**.** (from [FR1])

against invading pathogens. The response begins when antigen presenting cells (APCs) present an antigen, a pathogenic molecule in origin, to a subtype of T cell called a Helper T (T_H) cell. Then, depending on whether the antigen is viral, cancerous, or more macroscopic (bacterial or parasitic) in nature, either cytotoxic T (T_C) cells or plasma B cells are activated (Figure 1). T_C cells execute altered self cells, while plasma B cells secrete antibodies to target invading pathogens (Figure 1). In contrast to vertebrates, plants contain no adaptive immune system.

Adaptive responses cannot occur without an innate immune system for initiation. In vertebrates, phagocytes that patrol the circulatory and lymphatic systems engulf and break down pathogens into an array of antigens. These antigens are presented by APCs, which ultimately result in the activation of the adaptive immune response. Plants, and many invertebrates such as *C. elegans*, lack a circulatory and lymphatic system, which precludes them from possessing an adaptive immune system and necessitates their reliance on the innate immune system. However, all eukaryotes have an innate immune system to some degree.

Animals, plants, fungi, and even bacteria contain an innate immune system that employs methods of nonspecific defense. Examples of such defenses include physical barriers; such as epithelial skin cells in animals and the cortex or cuticle in plants, enzyme secretions that inhibit pathogenic infection, and if necessary, controlled apoptosis to prevent the spread of the pathogens. Though some animals contain motile leukocytes that attack invading pathogens, plants are incapable of supporting this type of innate defense. Instead, plants have evolved physical barriers, antimicrobial secretions, and specific proteins that are capable of recognizing particular proteins inherent to pathogens.

Plants are ancient organisms that have evolved over millions of years, and as result, have co-evolved alongside a variety of pathogens over the same duration of time. If plants lacked the ability to recognize particular pathogens, pathogens would have devised methods to circumvent the static

barriers of the innate immune system through evolution. This is not a plausible scenario as infection generally decreases the fitness of plants, and therefore, natural section would work to eliminate plants with undeveloped immune systems. Mutations that increase pathogen recognition and defense would quickly spread by natural selection. Thus, plants utilize forms of pathogen recognition to determine which pathogen is invading and how to respond accordingly.

Arabidopsis thaliana, a member of the mustard family of plants (Figure 2A), is capable of recognizing the bacterial pathogen *Pseodomonas syringae pv. DC3000* (Figure 2B and C). *P. syringae* is a rod shaped Gram-negative bacterium (Figure 2B), whose hosts include a wide range of plants. *P. syrinagae pv. DC3000* is a pathovar, short for pathogen variation, which infects DC3000 tomato plant and is also capable of infecting *A. thaliana.* A pathovar is denoted by '*pv.' s*ubsequent to the pathogen species' name and then, the species or genus that it infects following. Other strains of *P. syringae* do not utilize *A. thaliana* and do not infect it (O'Brien, et al., 2011). Despite the similarity of their genetic codes, *A. thaliana* is capable of distinguishing *P. syringae pv. DC3000* from other strains (O'Brien et al., 2011). This ability to recognize a specific pathogen while ignoring others suggests that two components for pathogen recognition exist.

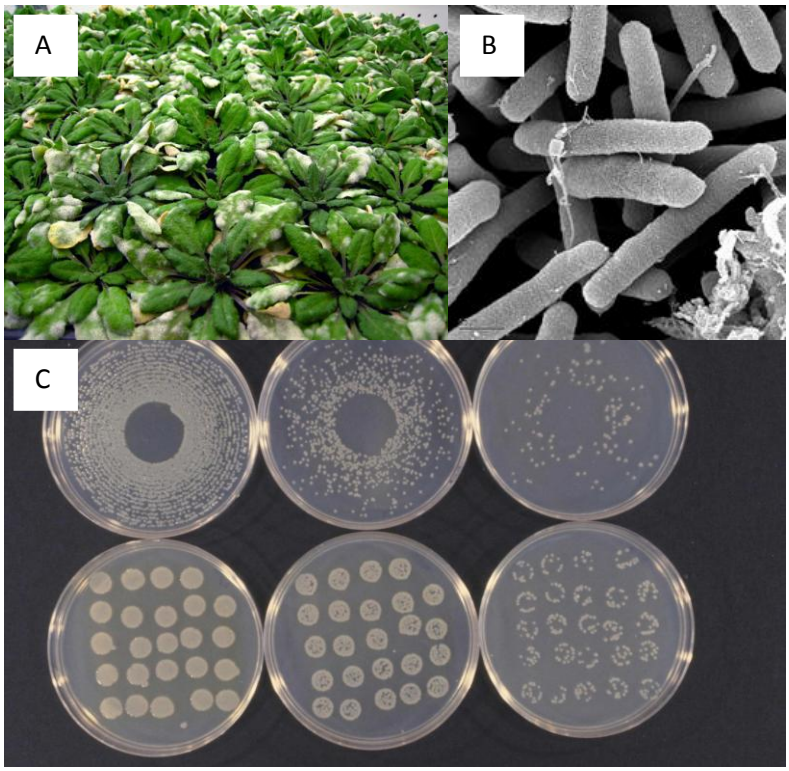

Figure 2 A) Maturing *Arabidopsis thaliana*. (from [FR2]) **B)** Electron micrograph of *Pseudomonas syringae*. (from [FR3]) **C)** Petri dishes containing colonies of *P. syringae.* (from Wikipedia user: Howard F. Schwartz)

First, the pathogen must contain a particular molecule that differentiates it from other pathogens whether of the same species or different species. Second, the host plant must contain some form of

receptor that specifically recognizes the unique protein of the pathogen. If either component were absent, no pathogen response would occur. These components are termed microbe-associated molecular patterns and pattern recognitions receptors.

Microbe-Associated Molecular Patterns

The first mentioned components necessary to elicit a response to a pathogen are microbe associated molecular patterns (MAMPs). MAMPs are often recognizable polypeptide regions of proteins produced by pathogens. MAMPs are sometimes referred to as PAMPs, but the usage of PAMP is slowly being phased out as the plant immune system will not only respond to molecules of pathogenic descent but also non-pathogenic microbial molecules. Thus, the term MAMP is favored because it encompasses any molecule produced by a microbe that triggers a defense response. Elicitation of MAMP responses by non-invading microbes is not limited to the laboratory environment in which various MAMPs are forcibly introduced to plant tissue and cell cultures. Anandalakshmi and colleagues (1998) demonstrated that plants lacking a particular P1/HC-Pro sequence in their DNA were more highly affected by synergistic infection. In their study, plants lacking this sequence that were normally immune to one virus became susceptible after being infected with the second virus. This suggests that while most non-invading microbes do not infect plants, there are certain circumstances in which harmless microbes become opportunistic pathogens.

MAMPs are similar to antigens that are recognized by the animal adaptive immune system, but differ with respect to recognition. Both MAMPs and antigens serve as respective identifiers for plants and vertebrates to mount an immune response to invading pathogens. However, MAMPs differ in the way they are recognized by a plant's innate immune system. In vertebrates, antigens are recognized by major histocompatibility complexes (MHC), T-cell receptors, and antibodies, which contain variable

regions that are altered through genetic recombination to bind epitopes of any conformation. Dissimilarly, MAMPs are recognized by non-variable receptors.

These receptors are termed pattern recognition receptors (PRRs) and are the second component required for pathogen recognition. PRRs are proteins with definite, immutable forms that are produced from genes that have not undergone recombination. Unlike T-cell receptors and MHCs, PRRs lack a variable region, and therefore, cannot recognize multiple MAMPs. The genes that produce PRRs do not undergo genetic recombination and are, therefore, fixed. Thus, PRRs are restricted to recognition of one particular MAMP. It should be noted that the MAMP recognition system that utilizes PRRs is highly conserved and exists in animals as well as fungi.

Generally, MAMPs are inherently linked to the fitness of pathogens, and removal results in marked decrease in survival. Motility, cellular structure, and protein production are all important to bacterial survival. To infect hosts, pathogens require motility to move from one host to another. When entering a new environment near or within the host, pathogens must be able to protect themselves from secreted antibiotics and differential pH. To protect themselves, bacteria rely on structural elements such as the cell wall. Prior to, during, and succeeding infection bacteria must be able to synthesize a complement of proteins to survive. All of these processes in pathogens are directly mediated or reinforced by MAMPs that can be detected by the plants immune system. Losing the MAMPs that mediate motility, contribute to cellular structure, or contribute to protein production respectively prevent the ability of pathogens to spread from one host to another, to defend against secreted antiobiotics and pH changes, and to reproduce. Losing any MAMP that mediates these processes is detrimental to a pathogen's ability to survive.

The protein flagellin is a common MAMP involved in bacterial movement (Felix et al., 1999). Movement in many bacterial species is facilitated by flagella (Figure 3), which are composed of the 20 nm protein known as flagellin. Though the entire flagellin protein acts as a MAMP, only a conserved 22 amino acid segment from the N-terminus is required for recognition (Felix et al., 1999). This region has been synthesized as 22 amino acid peptide called flg22. Flg22 is commonly used by researchers instead of flagellin as it elicits a similar strength response. Modification of this peptide can prevent detection as demonstrated by creating point mutations of the aspartic acid at position 43 from the N-terminus. The resulting D43V and D43A missense mutations rendered flagellin undetectable to the plant immune system (Naito et al., 2008). However, bacterial mobility was severely restricted.

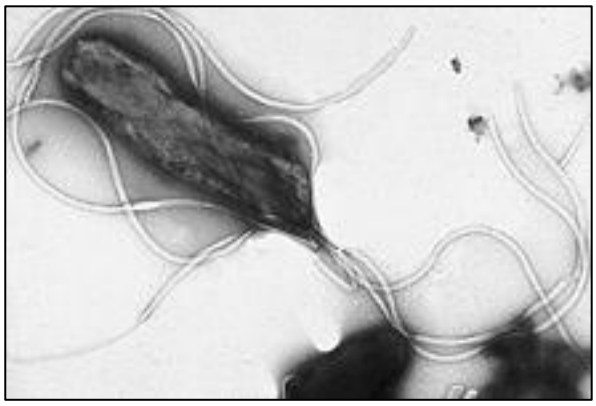

Figure 3 Bacterium Halicobacter pylori with its distinct flagella, which are comprised of flagellin proteins (from Yukata Tsutsumi, Department of Pathology, Fujita Health University School of Medicine)

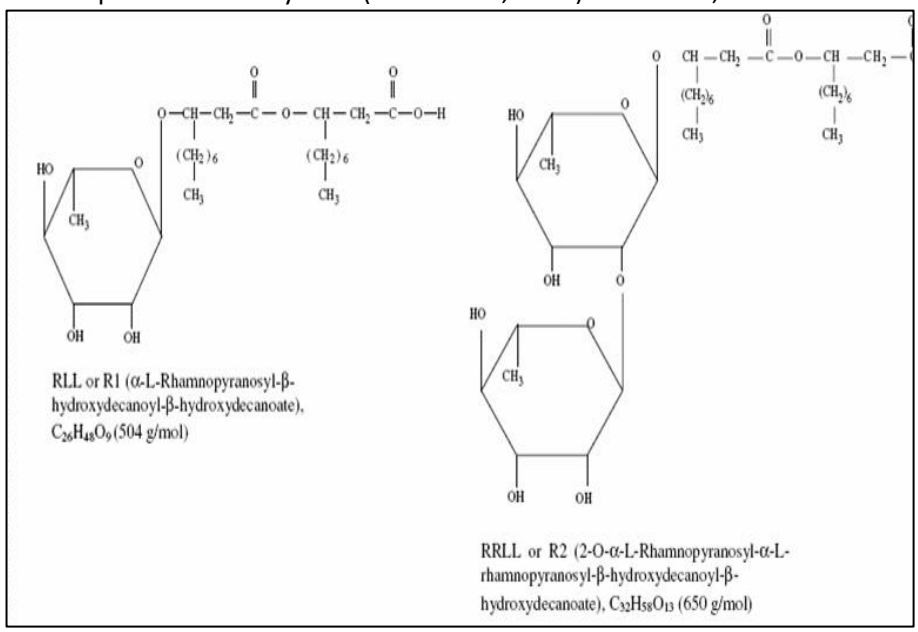

Figure 4 Example structures of two rhamnolipids. (adapted from Urum et al., 2006)

Survival of such bacteria outside of a laboratory setting would be greatly diminished, but their ability to avoid eliciting a response from the immune system (called a MAMP response) would be increased. In fact, it has been suggested that some bacteria shed their flagella prior to plant infection to avoid detection. It is known that the bacteria *Pseudomonas aeruginosa*, which

opportunistically causes infection in human burn victims, is able to shed the protein flagellin from its flagella. The mechanism by which this occurs is poorly understood, however, it has been found that amphoteric rhamnolipids initiate this process (Gerstel et al., 2009). Rhamnolipids are lipids exclusive to *Pseudomonas* bacteria. They have a particular polar sugar head called rhamnose and a non-polar fatty acid tail called 3-(hydroxyalkanoyloxy)alkanoic acid (HAA) (Figure 4). Rhamnolipids, like all lipids, are amphoteric because they can react as a base or an acid. Though this is currently the only model in which bacteria display this shedding capability, it suggests that other species like *Pseudmonas syringae*, which infects plants, may contain a similar flagellin dispensing mechanism.

Elongation Factor Tu (EF-Tu) is a prokaryotic protein that facilitates placement of aminoacyl-tRNAs into the free site of the ribosome and is also a MAMP (Figure 5). When EF-Tu transports tRNA to and binds the ribosome, EF-Tu undergoes a conformational change causing the hydrolysis of GTP (Villa et al., 2009).

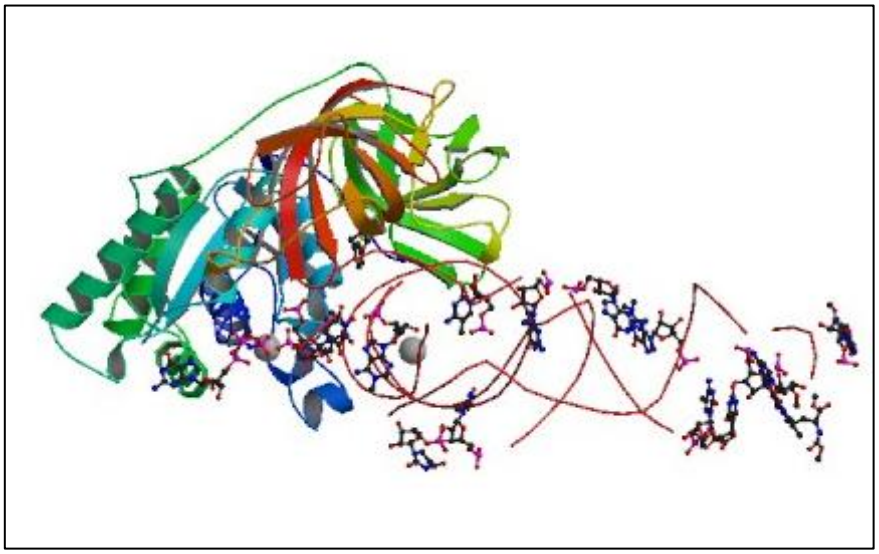

Figure 5 Ribbon diagram of elongation factor Tu coupled with phenyl-tRNA and GDP (adapted from Niseen et al., 1995; protein database: 1ttt)

The hydrolysis of GTP plays an essential role in one of the three methods of proof-reading translation (Dix and Thompson, 1986). The integral relationship of EF-Tu and its role in synthesis of new proteins within bacteria makes EF-Tu indispensable. Removal of this protein would prevent bacteria from assembling high fidelity proteins, which would not only waste cellular energy, but also detract from survival. Similar to the way in which flagellin contains a particular amino acid sequence that demarcates it as a MAMP, EF-Tu contains an 18 amino acid sequence in the N-terminus referred to as elf18

(Jeworutzki et al., 2010). Unlike flagellin, bacteria are incapable of shedding EF-Tu. In fact, this protein is often secreted from bacteria, which alerts the host plant to the bacteria's presence. However, EF-Tu detection has only been documented in *Brassicaceae* plants (Bent and Mackey, 2007).

In addition to flagellin and EF-Tu, there are other MAMPs that elicit lower level MAMP responses. One such MAMP is peptidoglycan (PGN). PGN is a polymer comprised of sugars and amino acids that participate in forming the cell wall of bacteria (Figure 6). The sugars that comprise PGN are

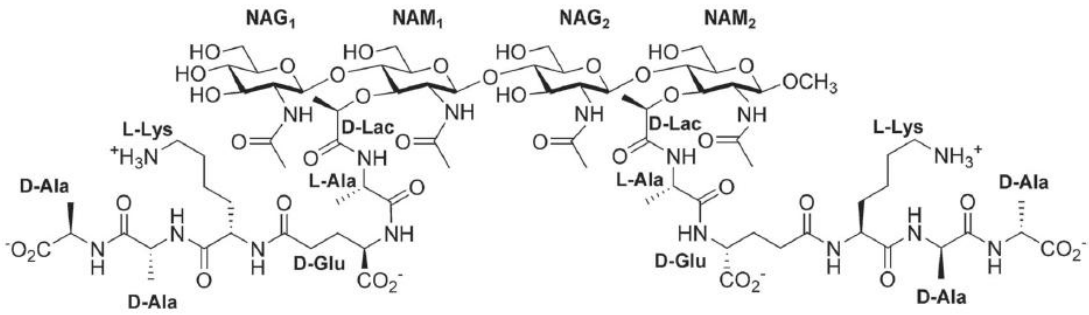

Figure 6 Structure of Peptidoglycan. Nam and Nag respectively refer to N-acetylmuramic acid and N-aceylglucosamine. (adapted from Meroueh et al., 2006)

N-acetlyglucosamine (Nag) and N-acetylmuramic (Nam) acid. These form β-(1,4)-glycosidic bonds, which result in the production of disaccharides that can be further strung together to form polysaccharides (Figure 6) (Meroueh et al., 2006; Erbs et al., 2008, Erbs and Newman, 2011). The formation of the cell wall occurs as a result of the enzyme transpeptidase, which facilitates cross-linking of amino acids of different PGN polysaccharides chains (Figure 8) (Meroueh et al., 2006; Erbs et al., 2008, Erbs and Newman, 2011). Like other MAMPs, PGN is required for pathogen survival. Without PGN, bacterial cell integrity is decreased, resulting in decreased survivability.

Another MAMP involved in providing structural support for bacteria is lipopolysaccharide (LPS) (Figure 7). As the name lipopolysaccharide suggests, LPS is a lipid and polysaccharide that are covalently bound. LPS is only found in gram negative bacteria and plays a vital role in reinforcing the cell wall, of which it is part, and protecting against certain chemicals that could otherwise kill the bacteria. Mutations in Wzx, Wzy, Wzz, WaaL, and/or MsbA, the proteins that synthesize and transport LPS (Wang and Quinn, 2010), result in poorly constructed LPS, causing bacterial death. LPS causes a strong immune reaction in animals, but a much weaker response in plants.

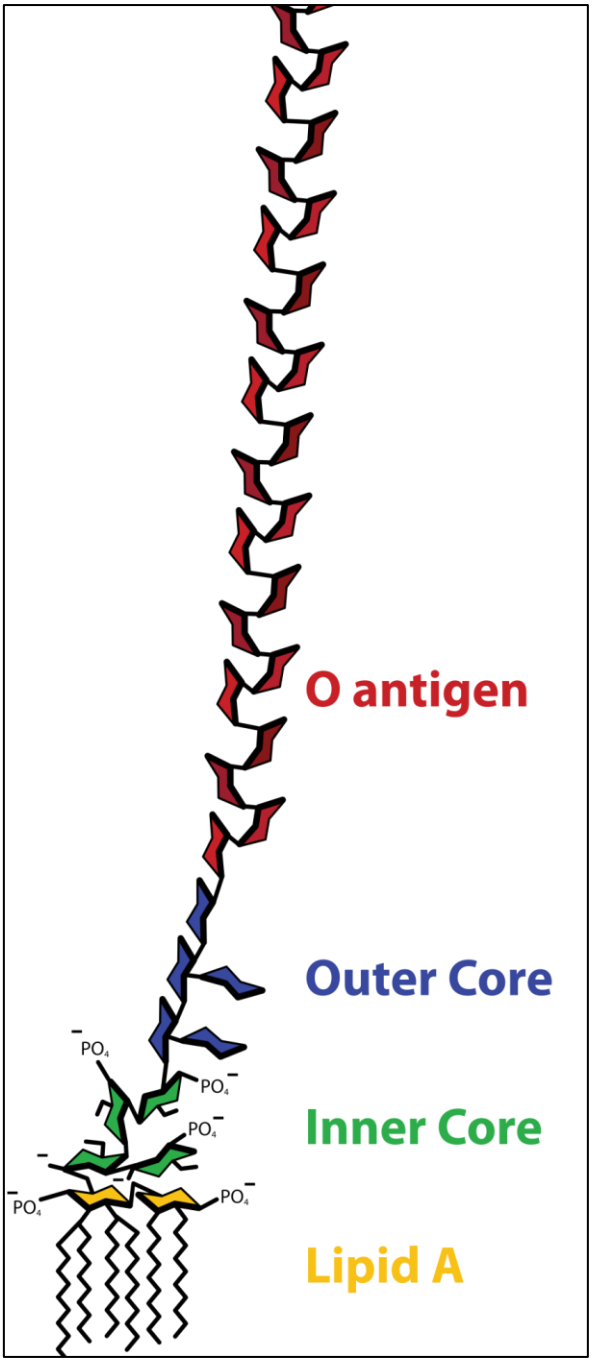

Figure 7 Schematized Lipopolysaccharide. The O antigen consists of repeating glycans. The outer core residue is short strand of sugars that vary between species of bacteria. Lipid A usually consists of a fatty acids attached to a phosphorylated glucosamine dissacharide. (from Wikipeida user: Mike Jones)

Both LPS and PGN elicit weak responses in plants as opposed to the stronger responses generated by flg22 and elf18. The differences in strength of response are suggested to result from MAMP charge and size (Aslam et al., 2009). Pectic acid, a component of the plant cell wall, lends a negative charge the exterior of plants (Morvan et al., 1984). When the negatively charged PGN and LPS interact with plants, they are repelled by and diffuse slowly through the negatively charged matrix of sugars that comprise the plant cell wall. Thus, it has been suggested that fewer molecules of LPS and PGN reach their respective PAMP receptors to trigger a PAMP response (Aslam et al., 2009).

Though charge plays a role eliciting MAMP responses, size is proposed to be the main factor. Large molecules such as PGN

and LPS have greater difficulty diffusing through plants' cell wall matrix. The movement of LPS is further complicated by its amphiphilic properties. An amphiphile is a molecule that is both hydrophilic (soluble in polar molecules) and hydrophobic (insoluble in polar molecules). It is this amphiphilicity that permit multiple LPS molecules to aggregate and form large structures with restricted movements through the cell wall (Gutsmann et al., 2007). The size of these aggregates is implicated in the production of a weaker, delayed PAMP response (Aslam et al., 2009). By contrast, elf18 and flg22, which consist of a little more than a dozen amino acids, easily traverse the 35 – 52 Å pores of the cell wall (Alving et al., 1979) and bind their respective receptors. Aslam and colleagues (2009) have noted that flagellin does not natively exist as a 22 amino acid polypeptide, but instead is a significantly larger 33kDa protein. However, the size of flagellin seems to impose few restrictions on diffusion through the plant cell wall. Irrespective of whether plants are exposed to flagellin or flg22, MAMP responses are inducible at subnanomolar concentrations (Felix et al., 1999).

In summary, MAMPs are bacterial proteins essential for a bacterium's fitness that are detected by plants. Flagellin, EF-Tu, and their corresponding peptides, as well as PGN and LPS elicit MAMP responses. The properties of these MAMPs in relation to the plant cell wall determine the degree to which MAMP responses occur. However, MAMPs are only one facet of a two part story. Though MAMPs are necessary to elicit a response, receptors tuned to recognize them must also be present.

Pattern Recognition Receptors

The briefly aforementioned pattern recognition receptors (PRRs) are the second component necessary for plants to exhibit an immune response. PRRs are plant receptors that directly interface with and bind MAMPs. Though it is known that PRRs are specific to each MAMP, only two PRRs have been identified (Zipfel, 2008). The status of a third, CERK1, is currently being deliberated in part because

its complementary MAMP is unknown (Gimenez-Ibanez et al., 2009). Known PRRs include FLS2 and EFR, which respectively bind and recognize flagellin and EF-Tu (Zipfel, 2008). The PRRs that bind peptidoglycan and lipopolysaccharides have yet to be discovered (Zipfel, 2008).

Flagellin-Sensitive 2 (FLS2) is the PRR complement that binds the MAMP flagellin. FLS2 is a transmembrane protein kinase that belongs to the protein subfamily XII (Gomez-Gomez and Boller, 2000). The crystalline structure of FLS2 has yet to be identified because the protein itself has not been isolated. Whether FLS2 exists as a monomer, homodimer, heterodimer, or other higher order structure prior to binding flagellin is unknown. Though the structure of FLS2 has yet to be elucidated, Dunning and colleagues (2007) extensively examined the active site of FLS2 to which flagellin binds. The suggested binding site of FLS2 is an extracellular leucine rich repeat (eLRR) domain (Figure 8) (Gomez-Gomez and Boller, 2000). As its name implies, the amino acid leucine plays a prevalent role in formation of LRRs. The consensus sequence of eLRRs in plants is xxLxxLxxLxxLxLxxNxL in which x is a variable amino acid, L is typically leucine but can be valine or isoleucine, and N is often asparagine but can be cysteine, threonine, or serine (Dunning et al., 2007; Matsushima et al., 2000). This consensus sequence was derived from the only plant protein with an elucidated eLRR structure, polgalacturonase-inhibiting protein (PGIP) (Dunning et al., 2007). However, this sequence is similar in construction to other LRRs (Matsushima et al., 2000), which are implicated as binding sites in a variety of proteins within plants and animals.

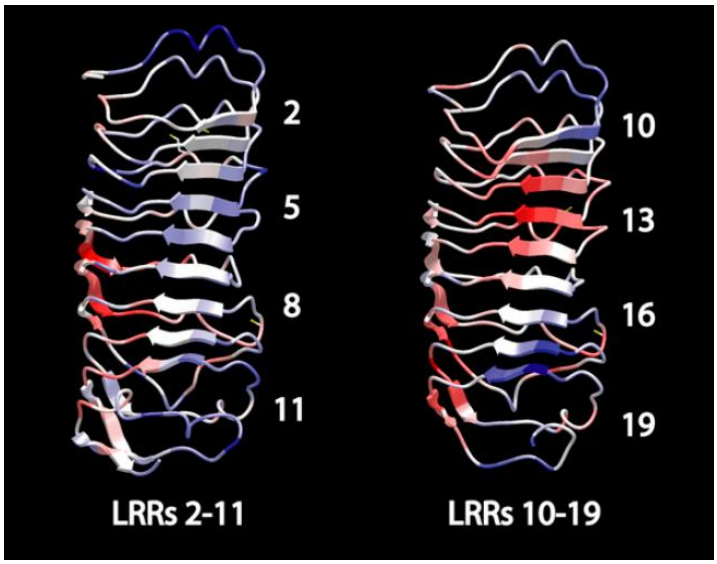

Figure 8 Estimated structure of what FLS2's LRR (adapted from Dunning et al, 2007)

Specifically, LRRs 12 through 14 of FLS2 (Figure 8) are implicated in binding flagellin (Dunning et al., 2007). This site was determined through mutational analysis of FLS2 by examining the fresh weight of mutant seedlings. Fresh weight is a common measurement used to determine allocation of resources within plants. Plants with nonfunctional FLS2 receptors have heavier fresh weight because they are unable to redirect resources to combat infection. Plants with functional FLS2 have a light fresh weight and demonstrate stunted growth. However, a plant's ability to combat infection should not be wrongly attributed to only FLS2. *Arabidopsis thaliana* ecotypes Ws-0, Dra-0, and Po-0 are sufficiently immunocompetent despite containing nonfunctional FLS2 alleles (Dunning et al., 2007). In general, FLS2 has been conserved across many plant species including the *Solanum* tomato plants and the *Solanaceae* plants *Nicotiana benthamiana* (Figure 9A) (Zipfel, 2008; Robatzek et al., 2007; Hann and Rathjen, 2007). *N. benthamiana* is indigenous to Australia and a relative of the tobacco plant. Plants that do not express

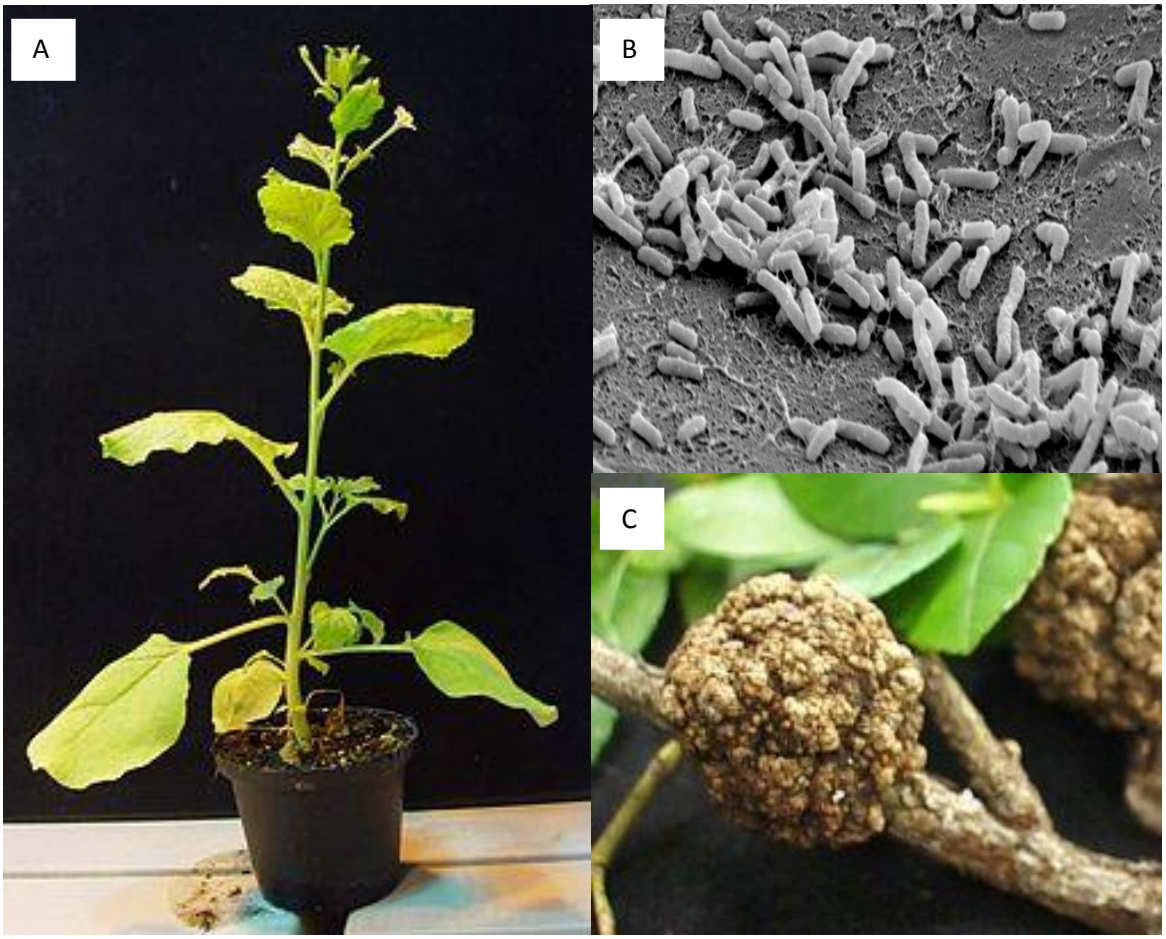

Figure 9 A) *Nicotania benthamiana* plant (from [FR4]) **B)** *Agrobacterium tumefaciens* (from [FR5]) **C)** Crown Gall caused by *A. tumefaciens.* (from [FR6])

FLS2 rely on another receptor called elongation factor receptor.

Elongation factor receptor (EFR) is a PRR that binds and identifies the MAMP EF-Tu. So far, responsiveness of EFR to EF-Tu has only been demonstrated in *Brassicaceae* species (Kunze et al., 2004). However, Zipfel and colleagues (2006) demonstrated that expression of the heterologous protein (*Arabidopsis thaliana* EFR) AtEFR restores responsiveness to elf18 peptides in *N.Benthamiana.* Zipfel and colleagues (2006) suggest that this demonstrates conserved downstream signaling pathways for MAMP responses. EFR, like the PRR FLS2, is part of protein subfamily XII (Nicaise et al., 2009; Zipfel et al., 2006). EFR contains a 21-LRR extracellular domain that has been suggested to serve as the binding site for the MAMP EF-Tu. However, the precise location of MAMP-binding has yet to be elucidated. EFR is a kinase with a cytosolic serine/threonine domain that is implicated in autophosphorylation upon activation by MAMP-binding (Nicaise et al., 2009; Xiang et al., 2008). Plants lacking EFR are more vulnerable to infection as demonstrated by Zipfel and colleagues (2006) when they infected *Arabidopsis* with A*grobacterium tumefaciens* (Figure 9B), a bacterium responsible for the production of crown galls (Figure 9C) in plants. *Arabidopsis* containing the EFR gene were significantly more resistant to transformation, while those that lacked EFR were considerable more susceptible to transformation. Zipfel and colleagues (2006) discovered that activation of both FLS2 and EFR trigger the same set of MAMP responses. This suggests the presence of protein that mediates the response of both EFR and FLS2. In fact, such a protein exists. Brassinosteroid insenstitive 1 (BRI1)-associated receptor kinase (BAK1) is a protein that perceives plant hormone brassinosteroids (BRs), but novel data also implicate it in mediating EFR signaling (Shan et al., 2008). While Shan (2008) and colleagues claim that BAK1 does not mediate the FLS2-flagellin response, Bent and Mackey (2007) present several sources in their review in which BAK1 appears to play a minor role in FLS2-flagellin signaling. Chinchilla and colleagues (2007) demonstrate that BAK1 does in fact bind EFR upon activation, but its purpose is unknown. Despite the conflict between the findings of other researchers and Shan and colleagues (2008) demonstrate that

removal of BAK1 from plant systems disrupts many MAMP-signaling pathways. One of these signaling pathways is EFR, which suggests that BAK1 is directly responsible for transducing signaling from EFR to other proteins to activate the necessary MAMP responses.

In summary, PRRs bind MAMPs, resulting in the transduction of a signal that will trigger a MAMP response and ultimately culminate in an immune response. Though each MAMP has a PRR with which it associates, only FLS2 and EFR, which respectively bind flagellin and EF-Tu have been discovered. FLS2 and EFR belong to a similar family of proteins and ultimately trigger the same MAMP responses. For this reason, it has been suggested that these two systems contain conserved and/or overlapping downstream elements.

Effectors and Resistance Genes

PRR-MAMP interactions are heavily studied to better understand the plant's basal immune system (MAMP defenses). However, the basal immune system is but one component of the plant immune system. A less understood, but equally well researched portion of the plant immune system is the gene-for-gene disease resistance schema. *Resistance* (*R*) genes produce a collective of protein products called resistance (R) proteins, which participate in identification of pathogen effectors. Effectors are pathogenic proteins that have been secreted into or gained entrance to the plant cell. As will be later discussed, the proposed dichotomy of effectors and MAMPs is unclear. The main defining feature of effectors is their location of action. Effectors most commonly operate within the host cell (Jones and Dangl, 2006; Bent and Mackey, 2007; Thomma et al., 2011). Additionally, a vast majority of effectors are not required for pathogen survival (Bent and Mackey, 2007). This attribute serves as a foil to MAMPs, which are necessary for pathogen viability (Felix et al., 1999; Bent and Mackey, 2007; Jones and Dangl, 2001).

Effectors are pathogenic proteins that interfere with pathogen recognition. Recognition interference can occur by disabling signaling initiated by PRRs or by interference with proteins that detect other effectors. Initially, effectors evolved to increase pathogenic virulence when invading hosts, but after years of co-evolution alongside their plant hosts, plants have developed R proteins that counter the effects of effectors (Chrisholm et al., 2005; Bent and Mackey, 2007). The ability to counter pathogenic virulence proteins has been termed gene-for-gene resistance as genes of plants counter genes of pathogens (Bent and Mackey, 2007). Effectors that are recognized by host R proteins detract from a pathogen's virulence as recognized effectors trigger immune responses that culminate in the activation of pathogen-resistance (PR) genes. PR genes produce proteins that defend against and prevention of further infection by pathogens. This triggers what is termed disease resistance. In some cases, a hypersensitive response (HR) is triggered and the cell senesces (Greenberg and Yao, 2004; Bent and Mackey, 2007; Jones and Dangl, 2001, 2006). For these reasons, recognized effectors are termed avirulence (avr) proteins.

The way in which effectors are recognized has spurred much debate among plant pathologists. The current functioning paradigm as to how R proteins recognize effectors is the 'Guard Hypothesis.' The guard hypothesis states that effectors are indirectly recognized by R proteins (Jones and Dangl, 2006). In other words, effectors are not identified by directly binding R proteins. Instead, effectors bind a target protein, which is modified and then recognized by R proteins. The archetypal system in which the Guard Hypothesis is best illustrated is the RIN4 system (Figure 10A and B). RIN4 is a 211 amino acid, acylated integral protein that is guarded by R proteins (Kim et al., 2005). RIN4 is implicated in the mediation of MAMP signaling, and therefore targeted by bacteria that would benefit from disabling MAMP responses. To date, there are three distinct effectors that target RIN4 and are recognized by R proteins in *Arabidopsis*. The unrelated AvrRpm1 and AvrB effectors are secreted into the cell (Figure 10A). Upon infiltration of the cell these effectors target RIN4 for phosphorylation of the threonine at

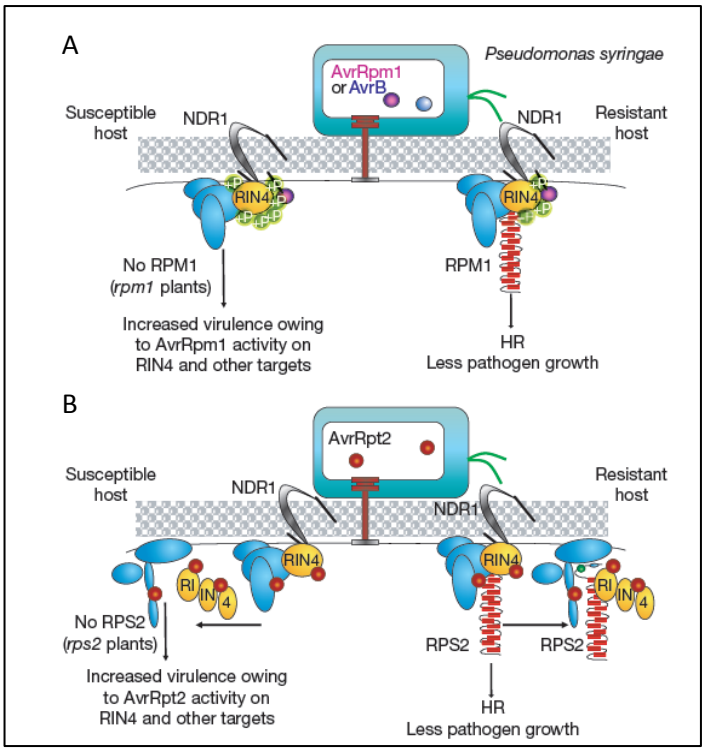

Figure 10 A) AvrRpm1 and/or AvrB are delivered through a bacterial Type Three Secretion System (TTSS). The effectors phosphorylate RIN4. RIN4's altered structure is detected by NB-LRR protein RPM1. **B)** AvrRpt2 is delivered through a TTSS and cleaves RIN4. The cleaved structure of RIN4 is recognized by NB-LRR protein RPS2, which signals a hypersensitivity response.(adapted from Jones and Dangl, 2006)

position 166 (Mackey et al., 2003; Jones and Dangl, 2006; Chung et al., 2011). Since neither effector is a kinase, this suggests that AvrRpm1 and AvrB take advantage of cellular machinery already present within the plant. Phosphorylation modifies RIN4, which is detected by *Arabidopsis* RPM1 nucleotide binding (NB)-LRR protein (Figure 10A). Detection results in elicitation of an HR and eventually senescence. It was discovered that replacement of threonine at position 166 results in constituent HR activation (Chung et al., 2011).

The third distinct effector is AvrRpt2. AvrRpt2, like the prior two effectors, is secreted into the cell, but unlike the other two, which rely on the plant cell's machinery to provide phosphorylation, AvrRpt2 is a cysteine protease that acts independently of the plant cell (Figure 10B). Cysteine proteases are enzymes that cleave peptide bonds to the amino acid cysteine. When AvrRpt2 contacts RIN4, it cleaves it in two particular locations, resulting in three separate peptides (Mackey et al., 2003; Jones and Dangl, 2006). Cleavage is recognized by RPS2 NB-LRR protein (Chrisholm et al., 2005; Kim et al., 2005; Jones and Dangl, 2006). Detection of cleavage ultimately results in decreased pathogen growth. In both effector-mediated processes RIN4 is the common target, which suggests that RIN4-mediated immune responses act through an adaptor protein that binds both RPM1 and RPS2 (Figure 10B). The adaptor protein implicated in binding these two proteins and facilitating disease resistance is NDR1 (Day et al., 2006). NDR1 is a glycophosphatidylinositol (GPI)-anchored protein, meaning that NDR1 is anchored to a

carbohydrate that is linked to a lipid by an inorganic phosphate group. GPI-anchored proteins may facilitate the formation of or direct the protein to microdomains in the plasma membrane called lipid rafts. Lipid rafts are extensively studied because of their implicated association with signal transduction and their high level of organization within plasma membrane.

Immune Responses in Plants

General Response: the Zigzag Model

Now that the key players have been identified, the actual disease resistance response can be examined. It is now widely accepted that two branches make up the plant immune system (Jones and Dangl, 2006). One branch consists of the transmembrane PRRs that identify MAMPs. This branch, referred to as the basal immune system, has evolved alongside and counters potential microbial invaders. The second is

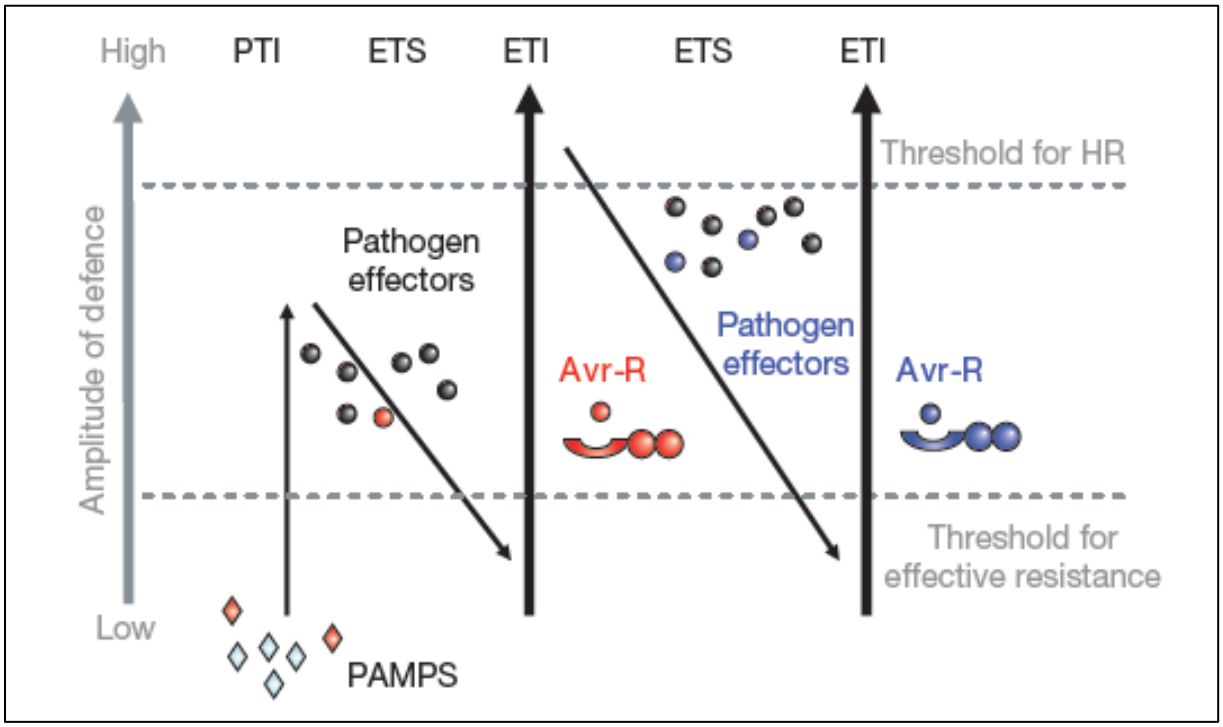

Figure 11 Zigzag model. MAMPs, written here is PAMPs, are detected and cause PAMP-triggered Immunity in plant cells. Pathogens release effectors that undermine the basal immune system, resulting in effector-triggered susceptibility. Effectors are recognized by R proteins, causing effector-triggered immunity. Depending upon the strength of the infection, a hypersensitivity response (HR) can be elicited, causing plant cell death. (Adapted from Jones and Dangl, 2006)

composed of polymorphic *R* genes that are translated into R proteins. R proteins are often identified by their NB-LRR domain, which is surmised to play a role in effector/modified host protein recognition. Though seemingly separate, these two systems are linked by the way in which pathogens interact with each system. These host-pathogen interactions have been named the zigzag model (Figure 11) as these interactions can be represented by four zigzagging phases.

During phase 1 the basal (PRR-mediated) immune system is the primary defense. Invading pathogens arrive on the scene and PRRs detect MAMPs, which result in PAMP-triggered immunity (PTI). This effectively prevents further infection. However, most pathogens contain a devious set of effector proteins. In phase 2, successful pathogens utilize effectors, which deactivate or allow microbes to circumvent PAMP defenses. The resulting condition plants suffer is effector-triggered susceptibility (ETS). ETS disposes plants to infection. In phase 3, plants counter ETS by deploying R proteins that directly bind via the R protein's NB-LRR domain or indirectly recognize its counterpart effector. Recognition of effectors by plants results in effector-triggered immunity (ETI). If pathogens cannot respond at this point, the infected plant cell undergoes a hypersensitive response, resulting in senescence (Jones and Dangl, 2006). In phase 4, pathogens can prevent senescence by down-regulating effector expression, ceasing production of or diversifying certain recognized effectors to suppress ETI. Pathogens capable of suppressing ETI return plants to a stage of ETS in which pathogens are once again capable of infection. Of course, by way of evolution, plants can diversify and modify their R proteins, which can help return plants a stage of ETI. The constant alteration between ETI and ETS has been described as an evolutionary arms race in which pathogens and plants vie for fitness. However, the intricacies of this arms race more closely resemble that of quick-paced tango in which different dance partners enter and leave the dance floor. In subsequent sections, the quick-paced tango known as the plant immune response will be described in more depth.

A Blurred Dichotomy

Though many reviews of the plant immune system tend to separate each phase and attempt to categorize responses, this will not be done here. While the above schema is useful for thinking about how plants sequentially respond to microbial threats, classification of immune responses into phasic responses is misleading. Like most immune systems, the plant immune system is cloaked in varying shades of gray instead demonstrating clear black and white delineations. Many MAMP responses elicited within plants are indistinguishable from R protein-facilitated responses (Thomma et al., 2011; Bent and Mackey, 2007). While the immune response may have been generated in response to either a PRR-MAMP interaction or and R protein-Avr protein interaction, responses become increasingly difficult to identify as MAMP responses and Avr responses interact with one another in down-stream signaling cascades. If differentiation was not difficult enough, Thomma and colleagues (2011) wrote a perspective article in which they discuss the "blurred PTI-ETI dichotomy" and partially attribute this blurring to the difficulty in defining MAMPs and elicitors. Thomma and colleauges (2011) state that highly conserved proteins which bind extracellular PRRs are typically considered MAMPs, while proteins belonging to a single or few related pathogen species that participate in triggering R protein responses are classified effectors (Chrisholm et al, 2006; Jones and Dangl, 2006; Bent and Mackey, 2007). However, some effectors are capable of being classified as MAMPs as well. As a result of this controversy, and the difficulty between differentiating, not only PTI and ETI, but also MAMP and elicitor, the phasic zigzag model will not be used to as a guideline. Instead, immune responses will be examined sequentially whenever possible, but due to many immune responses being concomitantly active while the plant is being besieged by pathogens, branching of pathways is bound to occur. However, each major response of the plant immune system will be examined as linearly and coherently as possible.

MAMP Responses

Plant PRRs bind and initiate responses to MAMPs. Upon binding, several immediate responses are induced. First, almost immediately, reactive oxygen species (ROS) are produced (Torres et al., 2006; Aslam et al., 2009). Second, nitric oxide (NO) is produced. Third, there is an influx of calcium (Ca^{2+}) (Aslam et al., 2009). Fourth, mitogen-activated kinases are activated (Chrisholm et al., 2006).

Reactive Oxygen Species

Upon pathogen invasion, reactive oxygen species (ROS) are produced. Reactive Oxygen Species (ROS) participate as signaling molecules to activate defense genes in plants. ROS are products of oxygen reduction and exist as superoxide ($\bullet O_2^-$), hydroperoxyl anion ($\bullet$HO2), hydrogen peroxide (H_2O_2), and/or hydroxyl radicals ($\bullet$OH). ROS production is cued when PRRs bind MAMPs and recognize invading bacteria. In *Arabidopsis*, FLS2 binds flg22, resulting in the subsequent binding of the receptor-like kinase BAK1. This FLS2/BAK1 complex in turn activates NADPH oxidases embedded within the membrane. NADPH, a respiratory burst oxidase homolog (rboh), then produces ROS, resulting in the biphasic accumulation of ROS in plant cells. The first phase is transient, which is succeeded by a second sustained phase in which ROS manage various cellular responses (Torres et al., 2006). At this juncture, it is not well understood whether the subsequent processes are independent and branching or linear. What is known is that ROS fulfills at least three roles related to pathogen resistance. First, ROS can directly strengthen the host cell walls by cross-linking glycoproteins (Figure 12) (Lamb and Dixon, 1997). Strengthening the cell wall slows the rate of and deters further infection. Second, ROS regulates HR (Figure 12). However, regulation differs with species as demonstrated with several gene knockouts. For example, the double mutant *A. thaliana rbohD* (*atrbohD)* and *atrbohF* genes in *Arabidopsis* decreased HR (Torres et al., 2002), and *N. benthamiana* with silenced *Nicotiana rboh* (*Nrboh*) genes suppressed HR

as well (Yoshioka et al., 2003). Interestingly, singly knocking out *atrbohF* actually increases HR and promotes resistance to pathogen infection (Torres et al., 2002). Finally, ROS are responsible for promoting the accumulation of salicylic acid (SA) (Figure 12). SA is a well-known molecule responsible for regulation of many processes within plants. During PAMP responses, SA is responsible for increasing production of PR-1 protein (Halim et al., 2009), which will be expounded upon during discussion of nitric oxide.

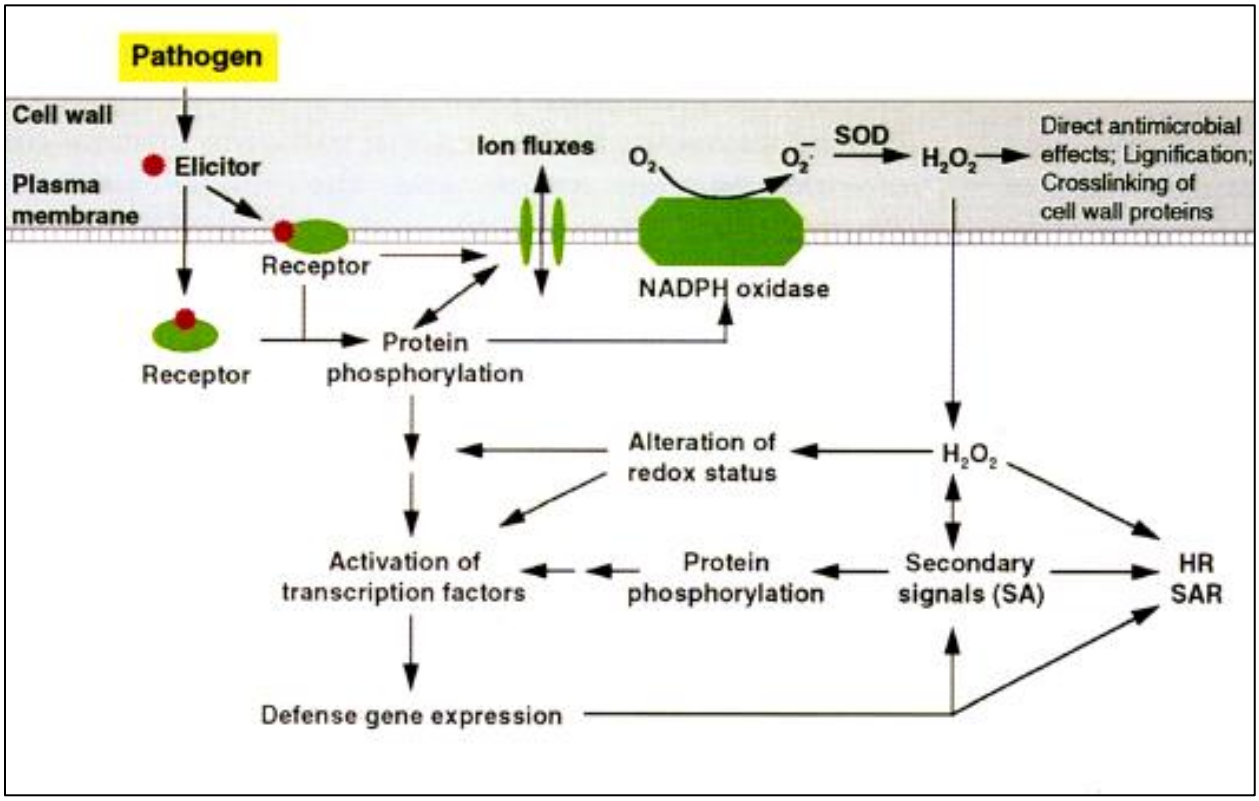

Figure 12 A simplified schematic of what an immune response. Pathogens trigger the response with an elicitor, commonly a MAMP, but sometimes an effector. Identification of the elicitor results in Ca^{2+}, oxidate bursts (ROS production), and initiation of kinase signaling pathways that activate PR genes. (adapted from Yang et al., 2012).

Calcium Influx and Reactive Oxygen Species

MAMP responses cause calcium (Ca^{2+}) influx (Aslam et al., 2009). Within the cellular environment, the Ca^{2+} anion is an important secondary signaling molecule. In many cellular systems Ca^{2+} plays a dual role. First, because it is a cation, an influx of Ca^{2+} can result in cellular membrane depolarization, which leads to other cellular changes. Second, Ca^{2+} binds the EF-hand domains of calcium modulating (CaM) proteins also called calmodulins. Binding CaM proteins results in conformational changes that initiate protein phosphorylation cascades in which phosphates are covalently attached to proteins. In plants, Ca^{2+} is intimately linked to ROS production (Figure 12). Rboh contain EF-hand domains that are capable of binding Ca^{2+} (Torres and Dangl, 2005), which result in the activation of Rboh and production of ROS. However, there is evidence indicating that Ca2+ influx occurs upstream of ROS production as well. Binding of flg22 by FLS2 and subsequent formation of the FLS2/BAK1 complex in *Arabidopsis* triggers activation of cation channels in the plasma membrane resulting in an influx of Ca^{2+} (Figure 12) (Jeworutzki et al., 2010). While it is accepted that this influx occurs independently of ROS activation, it has been hypothesized to partake in a positive feedback loop that prolongs ROS and Ca^{2+} accumulation. For example, FLS2/BAK1 promotes Ca^{2+} influx and ROS production which in turn causes more Ca^{2+}. If Ca^{2+} influx is also found upstream of ROS production, then the Ca^{2+} influx from FLS2/BAK1 binding might further reinforce ROS production, and therefore, increase Ca^{2+} influx. This mechanism of reinforcement might also explain the biphasic increases in ROS and the concentration of cytosolic calcium ($[Ca2+]_{Cyt}$) that are observed during MAMP responses (Figure 12).

Nitric Oxide

Similar to ROS, nitric oxide (NO) and its cognate reactive nitrogen intermediates also play a role in regulating the plant immune system. NO was previously considered a toxic molecule. However, after the discovery that nitric oxide and its cognate reactive nitrogen intermediates play a role as gaseous

signaling molecules, the way in which cellular signaling is examined has changed. Since discovering that NO acts as a signaling molecule, NO has been heavily studied in animal models. Plant researchers are also intrigued by the role of NO in plants. The primary question proposed by plant researchers is, "Does NO play a role in plant signaling, and if so, how closely is it related to animal system and what does it govern?"

NO's role in plants has only recently been elucidated, but thus far NO has been demonstrated to play an important role in regulating certain aspects of the plant immune system. When PRRs recognize PAMPs, an iNOS-like enzyme produces NO, and after one to two hours, the concentration of NO increases exponentially and peaks after approximately six hours. NO proceeds in regulating the immune system via two independent pathways. In the first pathway, NO modulates SA production. When SA production was prevented in transgenic plants possessing the *nahG* gene, very little SA accumulated despite the presence of NO production (Durne et al., 1998). This suggests that SA is an intermediate transduction molecule for NO. When SA accumulation occurs, pathogenesis related-1 (PR-1) protein is expressed.

In the second pathway NO up-regulates guanylyl cyclase's (GC) production of cyclic guanosine monophospate (cGMP). In animals, up-regulation of cGMP production occurs when NO binds a prosthetic heme group in GC forming a nitrosyl-heme. However, relatively recent evidence demonstrates that plants lack this heme group, which, in addition to demonstrating that NO in plants does not cause up-regulation of cGMP *in vitro*, precludes the possibility of direct regulation of cGMP by NO (Hong et al., 2008). Nitrosylation of an intermediate protein that then interacts with GC is the most likely explanation. Regardless of the mechanism, cGMP is up-regulated, which induces the synthesis of cyclic adenosine diphosphate ribose (cADPR). cADPR binds Ca^{2+} channels resulting in an increase of $[Ca^{2+}]_{Cyt}$. Then, through further signaling cascades, calcium induces transcription of phenylalanine lyase

(PAL) and PR-1 protein (Durneret al., 1998). Both proteins are involved in production of antimicrobials, and PAL is also involved in Nitrogen metabolism.

NO has also been implicated in initializing the hypersensitive response, which results in senescence. Both the hypersensitive response and regulation of immune system of plants are intimately linked. One of the main methods to deter invading pathogens is to cut off a pathogen's source of nutrients. When pathogens are detected, plants cut off nutrients through controlled cell death called senescence. For this reason, it seems logical that NO also mediate the hypersensitive response as well.

Kinase Signaling Cacades, Transcription Factors, and Disease Resistance

Mitogen-activated protein kinase (MAPK) signaling is induced in a MAMP response (Chrisholm et al., 2006). Kinases are a particular class of enzyme that transfer phosphate groups from high energy molecules like adenosine triphosphate (ATP) to other proteins via nucleophilic attack called phosphorylation (Appendix D, Figure S1). Phosphorylated proteins experience conformational changes that alter their activity, propagating signal transduction. MAPKs are a particular type of protein kinase that only phosphorylates hydroxyl groups of threonine and serine (Appendix D, Supplementary Figure S1). MAPKs are commonly responsible for signaling cascades that activate gene expression. In plants during infection, MAP kinases interact with WRKY transcription factors to modulate expression of proteins for fighting pathogens (Bent and Mackey, 2007). Subsequently, the specific mechanisms and roles of these responses will be examined in greater detail.

Ultimately, the goal of MAMP responses and effector-mediated responses is to activate disease resistance through PR genes (Figure 13 and 14). Activation of disease resistance is facilitated through downstream signaling that activates transcription factors, which allow for differential protein expression.

In many cases, signaling is achieved through kinase signaling cascades, which result in chains of proteins being phosphorylated.

In a highly generalized model of kinase cascade signaling, an external signal is transduced by a receptor from the outside of a plant cell to the inside. Multiple proteins may undergo phosphorylation before a mitogen-activated protein kinase (MAPK), also called an extracellular signal-receptor kinase (ERK), is activated. Upon activation, the MAPK may phoshporylate several other MAPK proteins, ending with a MAPK/ERK Kinase (MEK) that is translocated into the nucleus. Once translocation occurs, MEK may directly or indirectly act through another signaling cascade to activate a transcription factor. The transcription factor will then bind a particular region of DNA, resulting in the recruitment of RNA polymerase to transcribe a gene that will ultimately contribute to altering the function of the cell.

While kinase signaling is ubiquitous in eukaryotic cells, there are many derivations. Plant kinase signaling cascades are some of the most complex as the plant genome contains many redundancies within its genome (Figure 13) (Eckhardt et al., 2009; Takahashi, Tanase-Nicola, Pieter Rein ten Wolde, 2009). As a result, multiple proteins can fulfill the same or similar roles within plants.

Most kinase signaling cascades are initiated by MAMP recognition by a PRR. In one example, Pep13, a 13 amino acid oligopeptide fragment from the fungus *Phytophthora sojae*, is recognized by a yet to be discovered PRR. Upon recognition, there is a Ca^{2+} influx, ROS are produced, and a particular MAPK called an elictor-repsonsive MAPK (ERM) is activated (Ligterink et al., 1997). Ligterink and colleagues (1997) demonstrated that ERM was Ca^{2+}-dependent, but not dependent on the oxidative burst. This was determined by treating plants cells with anthracene-9-carboxylate (AC9), amphotericin B, and diphenylene iodonium (DPI), which respectively block ion channels, mimic MAMP-recognition-induced ion fluxes, and block ROS production. When ion channels were blocked, ERM was not activated, but when Amphotericin B was used to mimic Ca^{2+} influx, ERM was activated. By

contrast, when the production of ROS were precluded, ERM remained active, which suggests either that ROS production parallels ERM activation and is required for another intracellular process or ROS production occurs downstream of ERM activation. Irrespective of whether ROS production occurs as a result of ERM activation, ERM is translocated into the nucleus (Ligterink et al., 1997). The lack of a nuclear localization signal hints that ERM probably binds and alters the conformation of a transcription factor or protein thereby revealing a nuclear localization signal on the transcription factor that consummates the concurrent translocation of both proteins into the nucleus. This signaling cascade concludes when a transcription factor binds DNA at a PR gene, resulting in increased disease resistance.

Another kinase signaling cascade elucidated by Ishihama and colleagues (2011) functions similarly, but introduces the concept of concomitant protein activation. The cascade begins with MAMP recognition, which may facilitate aforementioned Ca2+ influx, ROS production, and NO production. However, the Ishihama and colleagues (2011) disregarded these already well-characterized MAMP responses, and instead, focused upon kinase signaling. Through a series of phosphylations MEK2 is phosphorylated, translocated into the nucleus, and phophorylates three functionally redundant MAPK homologs: salicylic acid-induced protein kinase (SIPK), wound-induced protein kinase (WIPK), and Ntf4. WIPK and SIPK are orthologs of the Arabidopsis MPK3 and MPK6, respectively. The protein Ntf4 shares 93.6% and 72.3% sequence identity with SIPK and WIPK, respectively (Ren et al., 2006). Thus, Ntf4 has been suggested to be functionally redundant with SIPK and WIPK in signaling the production of the anti-microbial chemical camalexin (Ishihama et al., 2011; Pitzschke et al., 2009; Ren et al., 2006). Ishihama and colleagues (2011) expand the redundancies of these proteins to other pathogen defense signaling pathways. Once MEK2 phosphorylates SIPK, WIPK, and Ntf4, these proteins phosphorylate WRKY8, a plant transcription factor, at serine-79 and serine-86 (Ishihama et al., 2011). Near the phosphorylation site, which is located by the N-terminus, a MAPK-docking site exists. This docking site is referred to as the D domain. Phosphorylation of WRKY8 is dependent upon the binding of SIPK, WIPK,

and Ntf4 to the D domain. Subsequent to binding and phosphorylation, WRKY8 binds a gene with a W box. The W box sequence is TTGACC/T, and precedes most genes to which WRKY transcription factors bind in response to external stresses like pathogen invasion. Binding to the W box concludes in the transcription of W box controlled genes and ultimately an increase in disease resistance (Ishihama et al., 2011). However, there is a minor addendum. Though SIPK, WIPK, and Ntf4 participate in the activation of WKRY8, it was demonstrated that WRKY8 is not the only transcription factor that regulates this increase in disease resistance. Silencing the presumed upstream WIPK, SIPK, and Ntf4 MAPKs resulted in a greater decrease in resistance against *A.tumefaciens* than when WRKY8 was silenced (Ishihama et al., 2011). Though it seems likely they would interact with similar proteins, no experiments involving individual silencing of SIPK, WIPK, or Ntf4 were reported. The difference in silenced proteins suggests that WIPK, SIPK, and Ntf4 branch and independently interact with other transcription factors. Once again, this underscores the complexity of plant kinase signaling.

At first it might seem beneficial to maintain MAPKs in a constitutively phosphorylated state regardless of whether bacteria are present, but in actuality, this can lead to inefficient energy expenditure. Part of the ongoing struggle for all organisms is budgeting energy consumption against the limited amount of resources which an organism can obtain. For plants, which are sessile for the duration of their life, energy cannot be obtained by moving from one location to another. Plants are restricted to the energy they receive from their immediate surroundings. For this reason, plants must regulate disease resistance. If plants constantly defended against pathogens, they would squander energy and risk failing to produce seeds or stunting their growth.

To prevent constitutive activation, plants negatively regulate defense responses with the MPK4 pathway. Plants with mutant *mpk4* exhibit a dwarf phenotype and high levels of ROS (Nakagami et al., 2006; Gao et al., 2008). Broderson and colleagues (2006) suggest that these peculiarities arise as the

result of an increase cellular concentration of SA. Similar phenotypes have been noted in *mekk1* and *mkk1/mkk2* double mutants (Gao etl al., 2008; Qiu et al., 2008a). These results have implicated MEKK1 and MKK1/2 as functioning MAPKs upstream of MPK4 in the MPK4 pathway. Thus, it has been suggested that the MEKK1-MKK1/2-MPK4 signaling cascade plays a putative role in negatively regulating H_2O_2 and SA production. Until it was discovered that MPK4 mutliphosphorylates the protein MAP kinase substrate 1 (MKS1) at serine 30 and 72, the molecular basis for this pathway's regulation could not be elucidated (Andreasson et al., 2005; Qiu et al., 2008). Prior to this discovery, the protein MKS1 had only ever been shown to interact with the transcription factor WRKY33 in yeast (Andreasson et al., 2005; Broderson et al., 2006). However, the function of this interaction was unknown. It is now understood that MKS1 is bound to WRKY33 and that MPK4 binds these two proteins to form a ternary complex. Upon binding, MPK4 phosphorylates MKS1, of WRKY33 (Figure 13) (Andreasson et al., 2005; Qiu et al., 2008b). When WRKY33 is freed from the ternary complex, it binds DNA, terminating in the transcription of PR genes that produce a SA hydrolase, which negatively regulates SA production. Interestingly, it has been found the MEKK1 also interacts with WRKY53, another transcription factor (Miao et al., 2007). This interaction allows for the MKK1/2, MPK4, and WRKY33 to be circumvented altogether (Figure 13). It has been suggested that WRKY53 exists as a shortcut to activate negative regulation (Miao et al., 2007; Pitzschke et al., 2009). It also is possible that this shortcut exists to avoid pathogen effector interference. As will be discussed, pathogens are capable of secreting effectors that can interfere with kinase signaling pathways to prevent disease resistance.

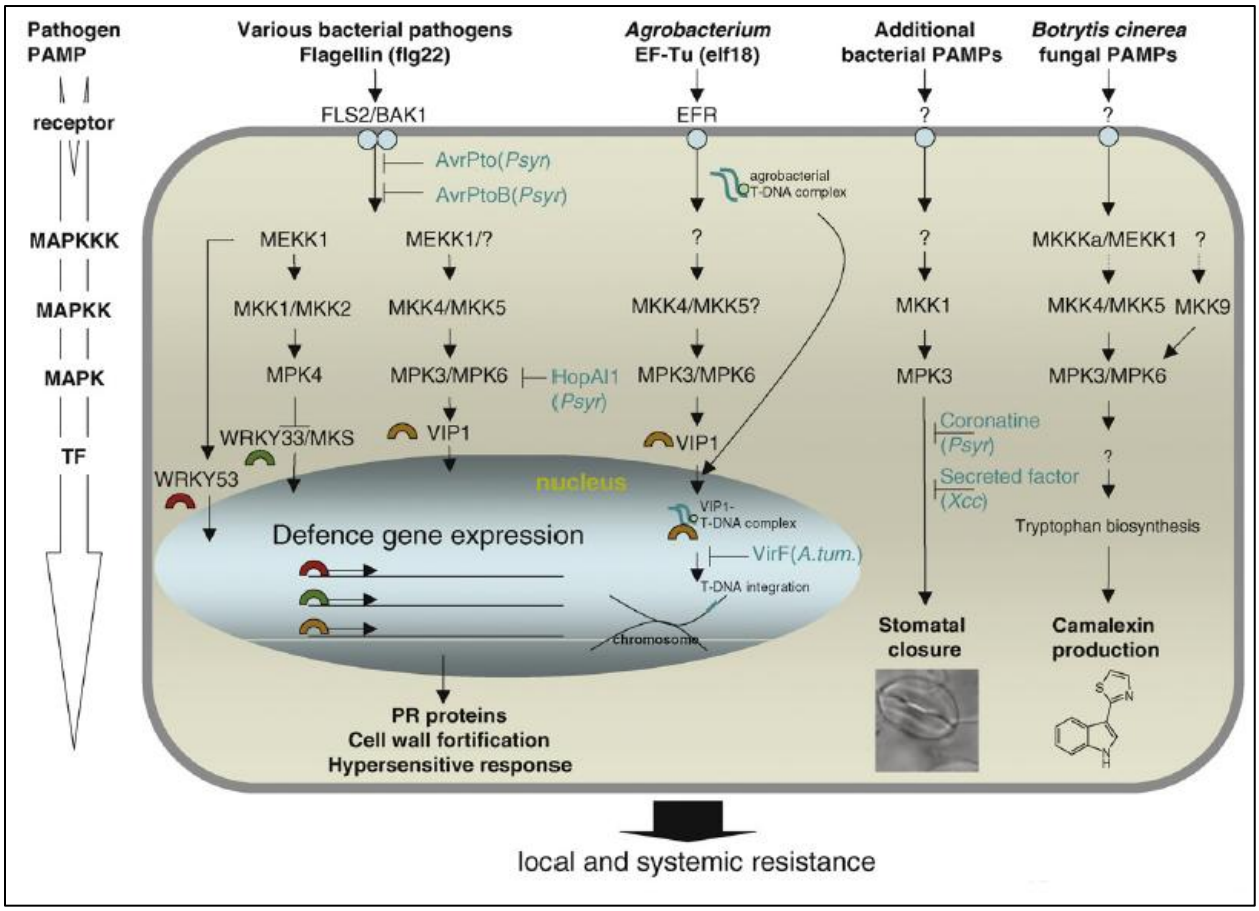

Figure 13 A schematic of the plant immune system signaling pathway. Most signaling pathways go through several phosphorylations of MAPKs before transcription factors are activated. The WRKY family of transcription factors is the most common transcription factor is plants with respect to signaling plant immunity. (adapted from Pitzschke et al., 2009)

In conclusion, kinase signaling is important for transducing messages that eventually result in PR gene transcription and disease resistance. Most often disease resistance is achieved through MAPK signaling cascades, which activate the plant family of WRKY transcription factors that modify gene expression. Constituent activation of kinase signaling is not conducive to survival as it hinders fitness. Therefore negative regulation of kinase signaling exists to check a potentially crippling system.

Effectors and Kinase Signaling

MAPKs are essential for activating the Pathogenesis-related (PR) genes that thwart infectious attempts. However, pathogens can interfere with kinase signaling via effectors. One of the most common targets for pathogenic effectors are PRRs. Disruption of PRR functionality precludes the possibility of PR genes being activated, and therefore, disease resistance.

FLS2 is targeted by the bacterial ubiquitin ligase AvrPtoB (Figure 14). Previous studies determined a decrease in the number of FLS2 proteins after infection by *P. syringae pv. DC3000* (Zwiesler-Vollick et al., 2002). Gohre and colleagues (2008) confirmed this finding through the use of FLS2-green fluorescent protein (GFP) hybrids. GFP is a 238 amino acid fluorescent protein that is commonly used as a reporter for protein expression. Plants were genetically modified to express FLS2-GFP hybrids and an inducible pathogen effector from *P. syringae pv. DC3000*. The effector was AvrPtoB. When AvrPtoB expression was induced the number of FLS2-GFP hybrids was decreased (Gohre et al., 2008). However, when MG132, a known proteasome inhibitor, was added, the decrease in FLS2-GFP was inhibited. This suggests that FLS2 undergoes AvrPtoB-mediated proteasomal degradation (Figure 17). Gohre and colleagues (2008) determined FLS2 is most likely targeted for degradation via ubiquination as multiple components are required for ubiquination. Removal of any component prevented ubiquination of FLS2. Thus, *P. syrinae pv. DC3000* was found to utilize the plant cell's own machinery against it to disable the PRR FLS2 signaling cascade.

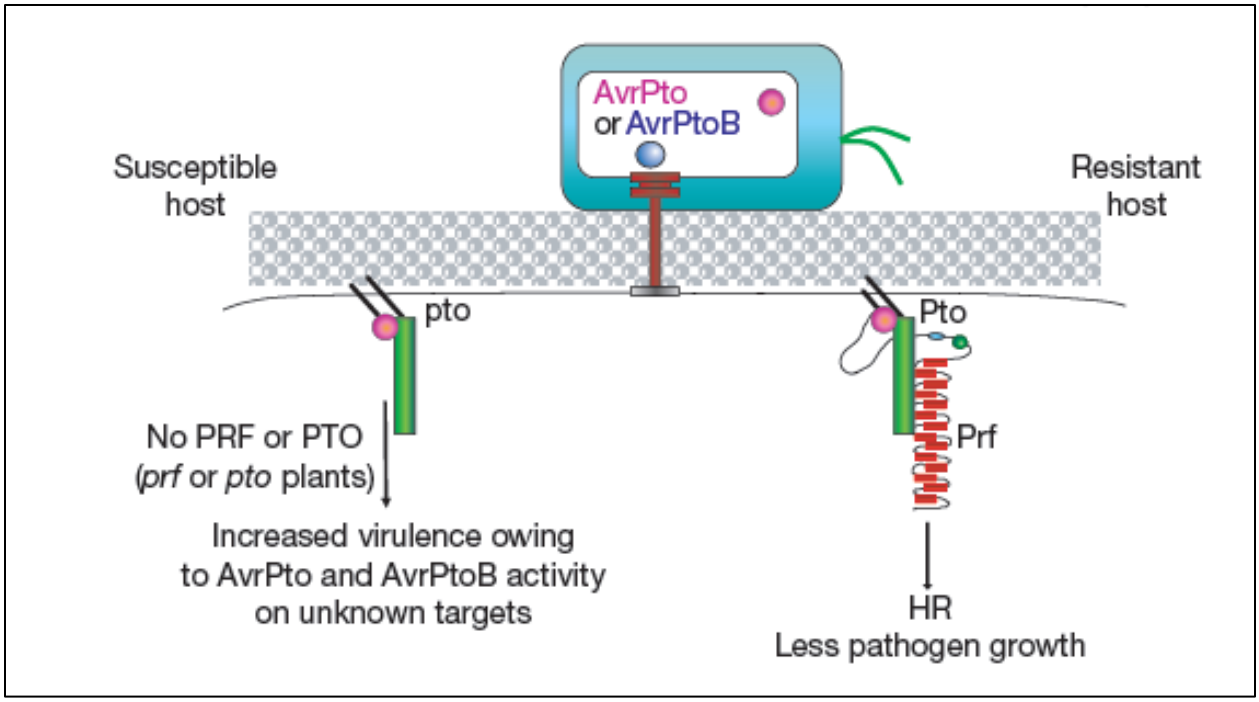

Figure 14 AvrPtoB or AvrPto are secreted into the plant cell with a type three secretion system. AvrPtoB targets FLS2 by ubuiquination and uses the plant's own proteasomes against it. (adapted from Jones and Dangl, 2006)

In another example in which pathogens interfere with kinase signaling, *A. tumefaciens* hijacks the MPK3/MPK6 signaling pathway. When *A. tumefaciens* encounters *A. thaliana*, it avoids FLS2 detection because it contains a modified flagellin sequence (Gomez-Gomez et al., 1999). However, *A. tumefaciens* appears to deliberately allow detection of EF-Tu, which normally activates VirE2-interacting protein 1 (VIP1) and culminates in the transcription of PR genes. *A. tumefaciens* takes advantage of its detection by hijacking VIP1 and using it to shuttle transfer-DNA (T-DNA) into the nucleus (Figure 13) (Citovsky etl al., 2004; Djamei et al., 2007). Once T-DNA enters the nucleus it is integrated into the host DNA where the T-DNA will be transcribed into enzymes that synthesize opines and phytohormones that can be used by *A. tumefaciens* as a source of nutrients. However, if VIP1 remains active, even though T-DNA will have been introduced, PR genes will have still been activated and the *A. thaliana* will be able to suppress the bacterial invasion. To bypass this potential caveat, *A. tumefaciens* secretes an effector called VirF, which gains access to the nucleus through an unknown mechanism and targets nuclear VIP1

for degradation (Tzfira et al., 2004). Execution of degradation is presumed to occur through a VirF domain called Skp1–Cdc53cullin–F-box (SCF) complex (del Pozo and Estelle, 2000; Tzfira et al., 2004).

The plant immune system is complex, but further elucidation could bring to fruition great advances in pathogen-resistance crops. However, before this possibility can become reality, further research must be conducted to understand how pathogens circumvent plants' immune systems. From unpublished research conducted by Anderson and colleagues (2011), it has been suggested that bacteria that have previously encountered a particular host may be better prepared to invade the same host if it is encountered again in the future.

The objective of this experiment was fourfold. First, optimize a reactive oxygen species (ROS) assay to measure a temporal MAMP response in *A. thaliana* leaves (Appendix F). Second, optimize an aequorin assay to measure a MAMP response in *A. thaliana* seedlings (Appendix F). Third, determine whether the bacterium *Pseudomonas syringae* is able to circumvent the immune system of *A. thaliana* if it has encountered *A. thaliana* before. Fourth, with preliminary data gleaned from each assay, determine the role of *mpk4 -/- A. thaliana* in the alteration of bacterial MAMP expression.

Material and Methods

Plant Material and Growth Conditions

Arabidopsis thaliana (Col-0 and aequorin) seeds were surface-sterilized with sodium hypochlorite (5%) and plated on half-strength Murashige and Skoog medium (Sigma-Aldrich). Seeds were stratified for at least 2 days at 4°C and germinated (Figure 15) aseptically at 24°C in a standard growth chamber (Percy) with a 12 hour/12 hour light/dark cycle for 2 –3 days.

Figure 15 7-day old Arabidopsis thaliana on plated on Murashige and Skoog medium (from [FR7])

After 5 – 7 days *Arabidopsis thaliana (*aequorin) seedlings were transplanted into soil in 4x6 plastic cartons and grown in a phytochamber under a 16 hour/8 hour light/dark cycle at 21°C/19°C for 3 weeks (Figure 16).

Figure 16 Phytochamber containing Arabidopsis thaliana and Nicotiana benthamiana. (from [FR8])

Exudate Production and Testing

Arabidopsis thaliana (Col-0 and mpk4 -/-) were grown at 24°C in a standard growth chamber (Percy) with a 12 hour/12 hour light/dark cycle for 5 –7 days. *Arabidopsis thaliana* seedlings were collected and submerged in 50mL distilled water for 24 hours to produce plant exudate. Plant exudates were tested for their quality by adding 100µL *P. syringae pv. DC3000* containing *LuxCDABE* gene (OD_{600} = 0.1) to 500µL plant exudate. Plant exudates that contain secreted molecules and/or proteins from

Figure 17 Photek camera (black) attached to a DB2 darkbox to prevent the interference of rogue photons. (from [FR9])

Arabidopsis thaliana cause bacterial bioluminescence (Peck, unpublished data). Bioluminescence was measured with a Photek HRPCS4 photon detection camera (Photek) (Figure 17).

Pseudomonas syringae Growth and Pretreatment

The bioluminescent strain of *P. syringae DC3000* containing *LuxCDEBA* operon was streaked onto a King's B agar (1.5g K2HPO4, 1.5g MgSO4•7H2O, 15g glycerol, 15g agar per 1000mL water). The pH of the solution was adjusted to 7.0 and autoclaved. Rifampicin (1mL/L) was added and *P. syringae* were grown at room temperature (Figure 18).

Figure 18 Pseudomonas syringae plated on agar. (from [FR10])

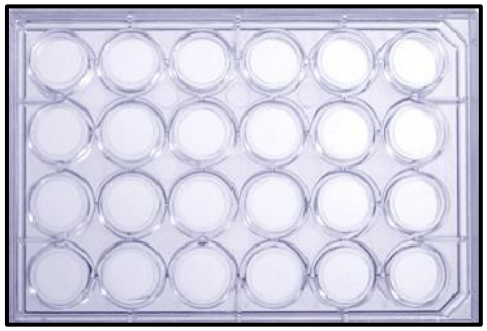

Figure 19 A 24-well plate in which Pseudomonas syringae were treated. (from [FR11])

After 3 days bacteria were scraped from the plate using a nichrome inoculation loop and were resuspended in 2.5mL distilled water. *P. syringae* were mixed with a micropipette to insure consistent distribution. 50mL of *P. syringae* were placed in 950mL of distilled water in a plastic 1mL cuvette. OD_{600} was measured. For *P. syringae* OD_{600} = 0.1 is the equivalent of $1x10^8$ bacteria/mL. *P. syringae* were diluted to a final concentration of $1x10^8$ bacteria/mL. 100µL of *Pseudomonas syringae were* pipetted into individual wells of 24-well plate containing either 0.5mL phosphate salts ($2Na_2HPO_4$:$3NaH_2PO_4$) and 0.5mL distilled water (–f), 0.5mL phosphate salts with fructose and 0.5mL distilled water (+f), 0.5 phosphate salts with fructose and 0.5mL and *Arabidopsis thaliana* (Col-0) exudate (w/+f), 0.5mL phosphate salts with fructose and 0.5mL and *Arabidopsis thaliana* (mpk4 -/-) exudate (m/+f) (Figure 19). Bacteria were incubated at in

a standard growth chamber 24° (Percy) for 4 hours, extracted, and centrifuged. The pellet and supernatant of *Pseudomonas syringae* were separated, collected, heated to 100°C on a heating block, and placed in a freezer at -80°C.

Imaging Arabidopsis thaliana (Col-0) Leaf Discs

Leaf discs taken from 3 week old *Arabidopsis thaliana* (Col-0) were halved and arrayed in a solid white 96-well plate with 50uL distilled water in each well (Figure 20A). Leaf discs were incubated at 24°C in a standard growth chamber (Percy) with a 12 hour/12 hour light/dark cycle for 24 hours. Distilled water was replaced with 50µL luminol (7mg/mL), 2µL horse radish peroxidase (220units/mg), and 20µL treatment (mock treatment (distilled water), elf26 (100nm), or *P. syringae* pretreated with either –f, +f, w/+f, or m/+f). Chemiluminescence of leaf discs was measured with a Veritas Luminometer (Turner Biosystems). Student's t test was used to determine the significance of elicited chemiluminescence with respect to the mock treatment.

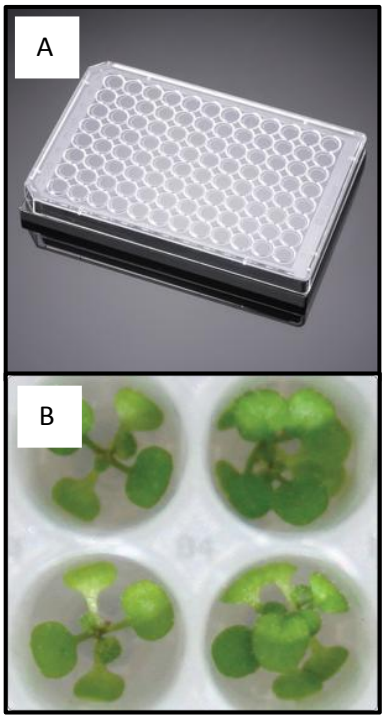

Figure 20 A) A 96-well plate in which leaf discs were arrayed. (from [FR12]) **B)** 2 - 3-day old Arabidopsis thaliana seedlings arrayed in the base of 200uL 96-well plate. (from [Fr13])

Imaging Arabidopsis thaliana (Aequorin) *Seedlings*

2 – 3 day old *Arabidopsis thaliana* (aequorin) seedlings were arrayed in a white plastic 96-well plate (Figure 20B) with 50µL Murashige and Skoog media and grown at 24°C in a standard growth chamber (Percy) with a 12 hour/12 hour light/dark cycle for 3 –4 days. Murashige and Skoog media was

removed and aequroin was reconstituted with 50µL coelenterazine (5µM) 24 hours prior to elicitation. Coelenterazine was replaced with 50µL calcium chloride (10mM) and 20µL treatment (mock treatment (distilled water), elf26 (100nm), or *P. syringae* pretreated with either –f, +f, w/+f, or m/+f). Bioluminescence was measured with a Photek HRPCS4 photon detection camera (Photek). Aequorin was discharged with 100µL discharge solution (2M $CaCl_2$ in 70% ethanol). Bioluminescence was measured as previously mentioned. Percent of total bioluminescence was calculated with the following formulation:

$$PTBL = \frac{MIBL}{TBL} \times 100\% ,$$

where $PTBL$ is percent of total bioluminescence, $MIBL$ is MAMP-induced Bioluminescence, and TBL is total bioluminescence. Student's t test was used to determine significance of elicited bioluminescence with respect to the mock treatment.

Results

The objective of this experiment was fourfold. First, optimize a reactive oxygen species (ROS) assay to measure a temporal MAMP response in *A. thaliana* leaves (Appendix F). Second, optimize an aequorin assay to measure a MAMP response in *A. thaliana* seedlings (Appendix F). Third, determine whether the bacterium *Pseudomonas syringae* is able to circumvent the immune system of *A. thaliana* if it has encountered *A. thaliana* before. Fourth, with preliminary data gleaned from each assay, determine the role of *mpk4 -/- A. thaliana* in the alteration of bacterial MAMP expression.

Reactive Oxygen Species Assay

A ROS assay was used to examine the MAMP response of *A. thaliana* to *P. syringae* that had been pretreated with *A. thaliana* exudate. Pelleted *P. syringae* and supernatant within which *P. syringae* had grown, were used to treat leaf circles. Figure 21 exhibits MAMP responses of *A. thaliana* treated with various pelleted *P. syringae*. In this experiment, the mock treatment suffered from a wounding response as seen from the high chemiluminescence with respect to other treatments (Figure 21 and Table 1). The mock treatment starts with high chemiluminescence and decreases steadily over time (Figure 21 and Table 1). By contrast, all other treatments demonstrate curves typical of MAMP responses (Figure 21). The 100nm elf26 treatment, which also appears to have suffered a wounding response, starts with high chemiluminescence and steadily increases to its peak after 9 minutes. Then, after 9 minutes, the MAMP response terminates, causing a steady decrease in chemiluminescence (Figure 21). All *A. thaliana* treated with bacterial pellet demonstrated MAMP responses of varying degrees (Figure 21). *A. thaliana* treated with *P. syringae* pretreated with only phosphates (-f) demonstrated the weakest MAMP response, starting with low luminescence and barely increasing after 8 minutes (Figure 21 and Table 1). The second lowest MAMP response was exhibited by *P. syringae* pretreated with phosphates and fructose (+f). *A. thaliana* treated with +f initially demonstrated low

chemiluminescence before slightly increasing chemiluminescence to an amount higher than –f after 9 minutes (Figure 21 and Table 1). *A. thaliana* treated with *P. syringae* pretreated with phosphates, fructose, and plant exudate from *A. thaliana* of ecotype col-0 (w/+f) demonstrated an intermediate MAMP response. The initial chemiluminescence of *A. thaliana* treated with *P. syringae* pretreated with w/+f was extremely low. However, nine minutes after treatment, chemiluminescence increased significantly before decreasing (Figure 21 and Table 1). The highest MAMP response not elicited by the purified MAMP elf26 occurred in *A. thaliana* exposed to *P. syringae* that been pretreated with phosphate, fructose, and exudate of *A. thaliana* with MPK4 knockouts (m/+f). *A. thaliana* treated with *P. syringae* pretreated with m/+f demonstrated high levels of initial chemiluminescence, which significantly increased after nine minutes (Figure 21 and Table 1).

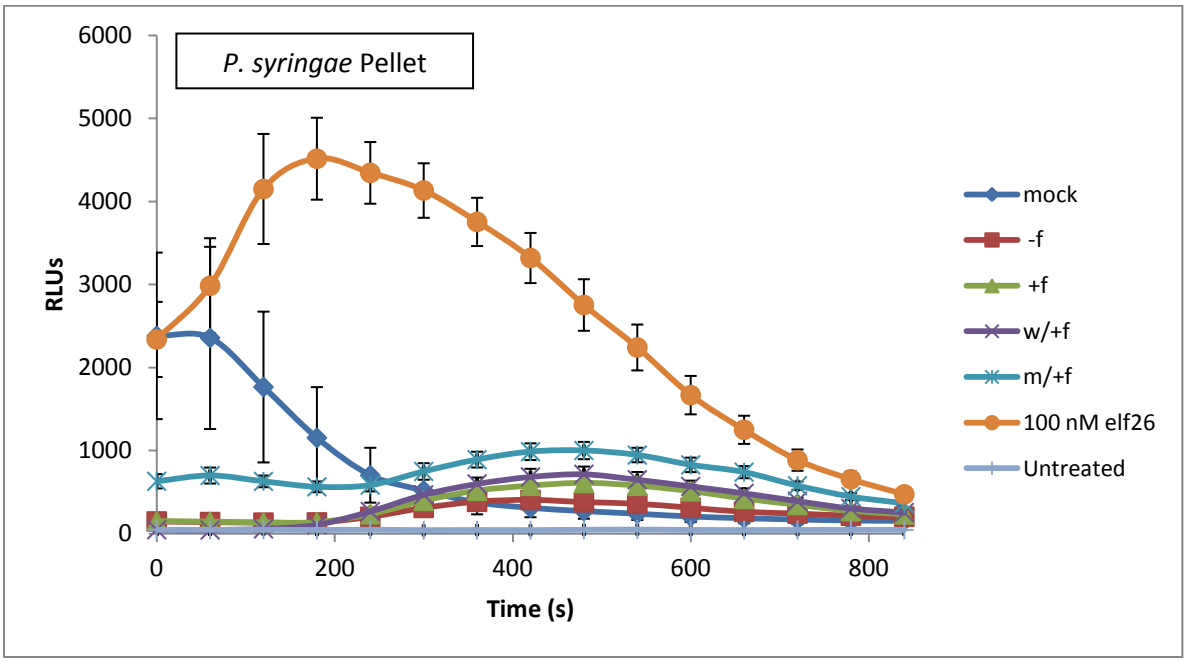

Figure 21 Chemiluminescence emitted by luminol as a byproduct of ROS formation during MAMP responses of A. thaliana untreated or treated with mock treatment, elf26, or pelleted *P. syringae* pretreated with –f, +f, w/+f, m/+f. Error bars are standard error.

	Chemiluminescence (RLUs)						
Time (s)	mock	-f	+f	w/+f	m/+f	elf26	Untreated
0	2456.40	82.31	103.61	9.78	923.70	1108.51	7.92
60	2687.43	81.38	109.64	12.62	1083.20	1403.14	11.15
120	2224.23	72.95	102.85	15.92	954.77	1623.21	8.73
180	1495.85	68.38	85.53	26.01	791.73	1208.85	6.96
240	809.20	105.50	125.45	92.92	667.07	910.11	4.86
300	553.88	159.68	195.96	149.18	616.04	804.35	3.46
360	354.35	193.56	218.60	187.87	589.05	711.56	11.09
420	274.65	191.20	250.77	239.12	533.27	739.42	7.17
480	225.42	155.47	229.66	227.77	468.73	760.27	6.39
540	183.50	128.78	202.53	229.00	403.07	676.74	17.84
600	161.82	142.14	186.38	170.05	336.85	566.78	12.63
660	145.96	132.20	148.84	143.78	286.61	415.49	12.58
720	139.59	136.44	110.88	98.42	205.42	314.16	5.17
780	141.53	107.74	87.68	54.97	159.29	227.74	9.66
840	137.85	111.35	79.38	46.29	122.83	146.10	5.87

Table 1 Exact measures of chemiluminescence (in relative light units) emitted during the MAMP response of *A. thaliana* untreated or treated with mock treatment, elf26, or pelleted *P. syringae* pretreated with –f, +f, w/+f, m/+f. Values correspond to those plotted in Figure 21.

The supernatant in which pretreated *P. syringae* were grown, were also used to treat *A. thaliana* leaf circles. Figure 22 exhibits MAMP responses of *A. thaliana* treated with various supernatants in which *P. syringae* were grown. In this experiment, *A. thaliana* treated with the mock treatment suffered a weak wounding response, and thus, began emitting low chemiluminescence that gradually decreased over time (Figure 22 and Table 2). *A. thaliana* treated with the supernatant of *P. syringae* pretreated with +f and elf26 respectively exhibited little and no wounding response, while *A. thaliana* treated with *P. syringae* pretreated with w/+ and m/+ both suffered a relatively high wounding response (Figure 22 and Table 2). Elf26 exhibited a typical MAMP response in which *A. thaliana* treated with elf26 demonstrated low levels of chemiluminescence that increased to high levels after 210 seconds before the MAMP response end and chemiluminescence decreased (Figure 22 and Table 2). In contrast to elf26, which demonstrates a normal MAMP response, *A. thaliana* treated with *P. syringae* pretreated with –f, +f, w/+, and m/+ all demonstrated a linear increase of RLUs over time (Figure 22). This increase

is a non-standard MAMP response as MAMP responses are characterized by their bell-shaped cruve. *A. thaliana* treated with *P. syringae* pretreated with +f and –f demonstrated the weakest MAMP response, while *A. thaliana* treated with *P. syringae* pretreated with w/+ and m/+ represented the two strongest MAMP responses (Figure 22 and Table 2). *A. thaliana* treated with *P. syringae* pretreated with m/+ had the strongest overall MAMP response (Figure 22 and Table 2).

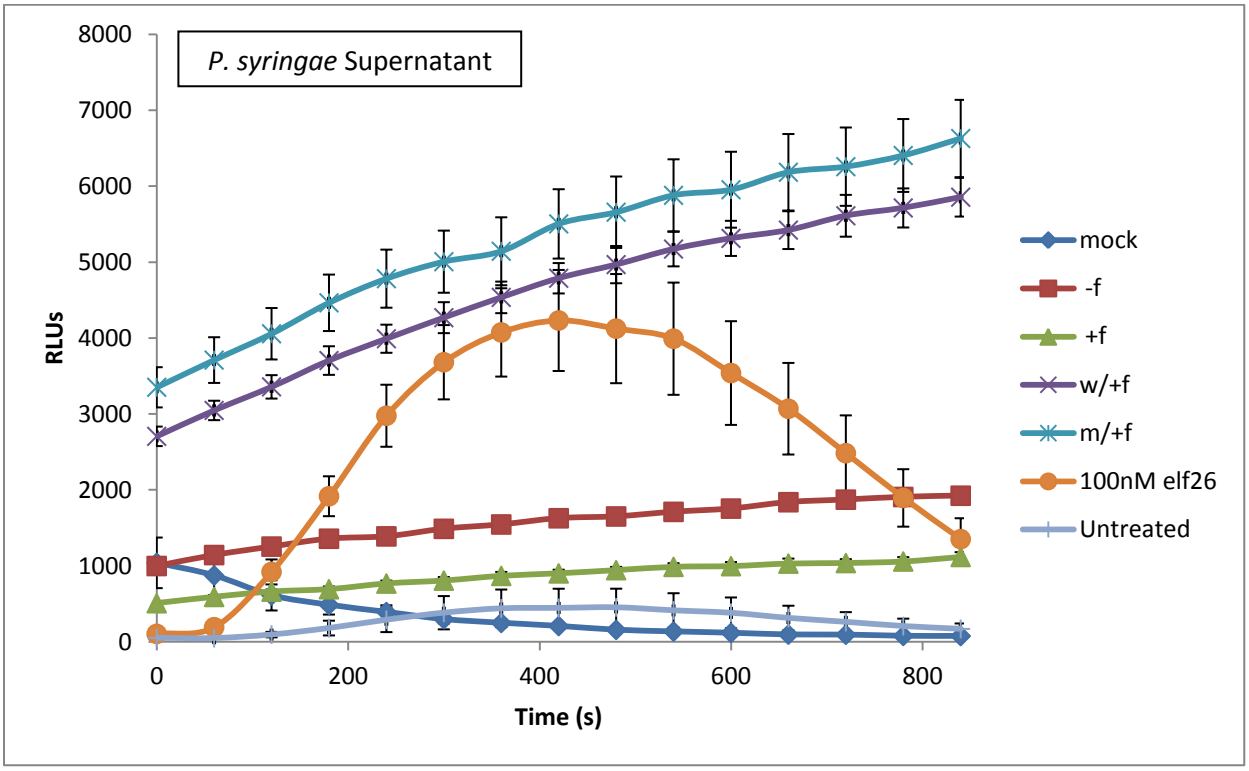

Figure 22 Chemiluminescence emitted by luminol as a byproduct of ROS formation during MAMP responses of A. thaliana untreated or treated with mock treatment, elf26, or the supernatant of *P. syringae* pretreated with –f, +f, w/+f, or m/+f. Error bars are standard error.

	Chemiluminescence (RLUs)						
Time (s)	mock	-f	+f	w/+f	m/+f	100nM elf26	Untreated
0	1041.17	999.17	509.17	2706.83	3352.50	105.50	52.27
60	874.67	1143.33	592.17	3047.83	3711.00	193.50	49.53
120	612.67	1254.67	662.00	3358.33	4057.67	921.50	96.03
180	487.33	1358.50	693.67	3704.50	4464.83	1918.00	182.23
240	393.17	1389.17	768.50	3992.00	4782.83	2977.83	291.20
300	298.50	1488.50	807.33	4269.33	5006.66	3682.50	384.27
360	249.67	1546.33	869.33	4536.50	5143.67	4075.00	441.27
420	208.50	1628.17	902.33	4788.50	5504.00	4232.50	447.20
480	158.17	1651.50	944.67	4967.83	5657.83	4125.17	454.67
540	136.17	1711.83	988.17	5172.50	5880.33	3992.33	414.83
600	118.33	1755.17	997.83	5313.33	5954.17	3540.17	382.03
660	95.83	1839.33	1031.17	5422.00	6183.67	3070.17	314.90
720	94.17	1872.67	1038.50	5610.83	6257.67	2484.67	262.77
780	78.00	1908.33	1058.83	5714.00	6404.83	1895.83	207.53
840	76.66	1924.67	1118.67	5855.67	6628.33	1352.67	170.93

Table 2 Exact measures of chemiluminescence (in relative light units) emitted during the MAMP response of *A. thaliana* untreated or treated with mock treatment, elf26, or pelleted *P. syringae* pretreated with –f, +f, w/+f, m/+f. Values correspond to those plotted in Figure 22.

Aequorin-Calcium Assay

An aequorin-calcium assay was used to examine the MAMP response of *A. thaliana* seedlings treated with *P. syringae* pretreated with *A. thaliana* exudate. Figure 23 exhibits cumulative bioluminescence resulting from treatment of *A. thaliana* with *P. syringae* pretreated with *A. thaliana* exudates. As seen in figure 23A, it appears as though *A. thaliana* treated with *P. syringae* pretreated with –f bioluminesced the most (Figure 23A: columns 5 and 6). However, this is not consistent with figure 24, which shows that *A. thaliana* seedlings treated with *P. syringae* pretreated with –f demonstrated the least bioluminescence and that those treated with *P. syringae* pretreated with m/+f most significantly bioluminesced despite appearing to have low luminescence in figure 23A. However, Figure 23B demonstrates that all treatments contain the possibility of bioluminescing at high levels. In fact, *A. thaliana* treated with *P. syringae* pretreated with –f contain much higher levels of bioluminescence when discharge solution is added (Figure 23B). This means that overall *P. syringae* pretreated with –f

elicited less bioluminescence than *P. syringae* pretreated with m/+f. The discrepancy between these data can be explained primarily as a result of variability within the same *A. thaliana*. Figure 23A in comparison to figure 23B demonstrates the discrepancies were within the same *A. thaliana* plant and was used to normalize data.

A B

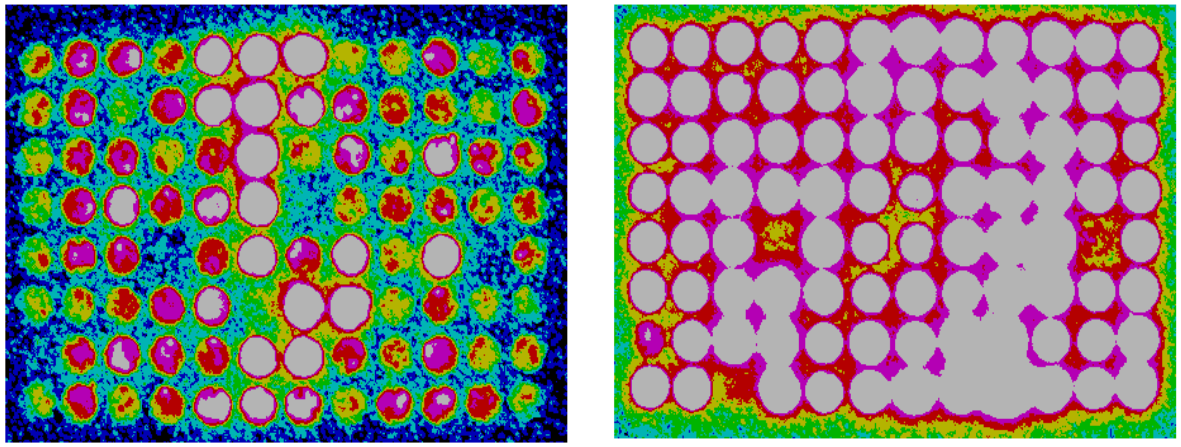

Figure 23 False heat map generated by cumulatively recorded photon emissions during aequorin bioluminescence. The number of photon emissions roughly correspond to color in the heat map (black = absent, blue = lowest, green = low, yellow = moderate, red = moderately high, pink = high, white = highest). Bioluminescence of aeuqorin-coelenterazine complex in response to calcium influx initiated during a MAMP response of *A. thaliana* treated with mock treatment (column 1 and 2), elf26 (3 and 4), or pelleted *P. syringae* (odds) or supernatant of *P. syringae* (evens) pretreated with –f (5 and 6), +f (7 and 8), w/+f (9 and 10), or m/+f (11 and 12). A) MAMP-induced bioluminescence. Elapsed time t = 14 minutes. B) Total bioluminescence acquired from addition of Ca^{2+} discharge solution. Elapsed time t = 14 minutes.

Though there were discrepancies within the same *A. thaliana*, there was also much variability between samples in the same treatment (Figure 23A). While *A. thaliana* treated with the bacterial pellet of *P. syringae* provide fairly consistent results, there are a few outliers within each treatment. Trial 7 of column 1 of mock treatment, trial 2 column 3 and trial 5 column 4 of the elf26 treatment, trial 6 of the supernatant of *P. syringae* pretreated with –f, trial 4 of the pelleted *P. syringae* pretreated with the +f, and trial 5 of pelleted *P. syringae* pretreated with m/+f are all outliers that exhibit unnaturally low bioluminescence with respect to other trials within their treatment group. By contrast, trials 5 and 6 of the supernatant of *P. syringae* pretreated with +f, trials 3 and 5 of supernatant of *P. syringae* pretreated

with w/+f are outliers that exihibt excessive bioluminescence with respect to other trials in their treatment group.

Figure 24 displays the results of treating *A. thaliana* with pelleted *P. syringae*. *A. thaliana* seedlings treated with a mock treatment expressed a low bioluminescence (Figure 24). *A. thaliana* seedlings treated with the MAMP elf26 bioluminesced significantly higher ($p < 0.01$) than the mock treatment (control) (Figure 24). Seedlings treated with *P. syringae* pretreated with *A. thaliana* pretreated with –f expressed significantly higher ($p < 0.01$) luminescence than the control (Figure 24). *A. thaliana* seedlings treated with *P. syringae* pretreated with +f bioluminesced significantly higher ($p < 0.05$) than the control. *A. thaliana* treated with *P. syringae* pretreated with w/+f bioluminesced lower than *A. thaliana* treated with *P. syringae* pretreated with either –f or +f. It too expressed bioluminescence significantly higher ($p < 0.01$) than the mock treatment. Seedlings treated with *P. syringae* pretreated with m/+f luminesced significantly higher ($p < 0.05$) than the control. Of the four bacterial pellet-based treatments, -f pretreated bacteria elicited the greatest bioluminescence from *A. thaliana* seedlings and m/+f elicited the least bioluminescence.

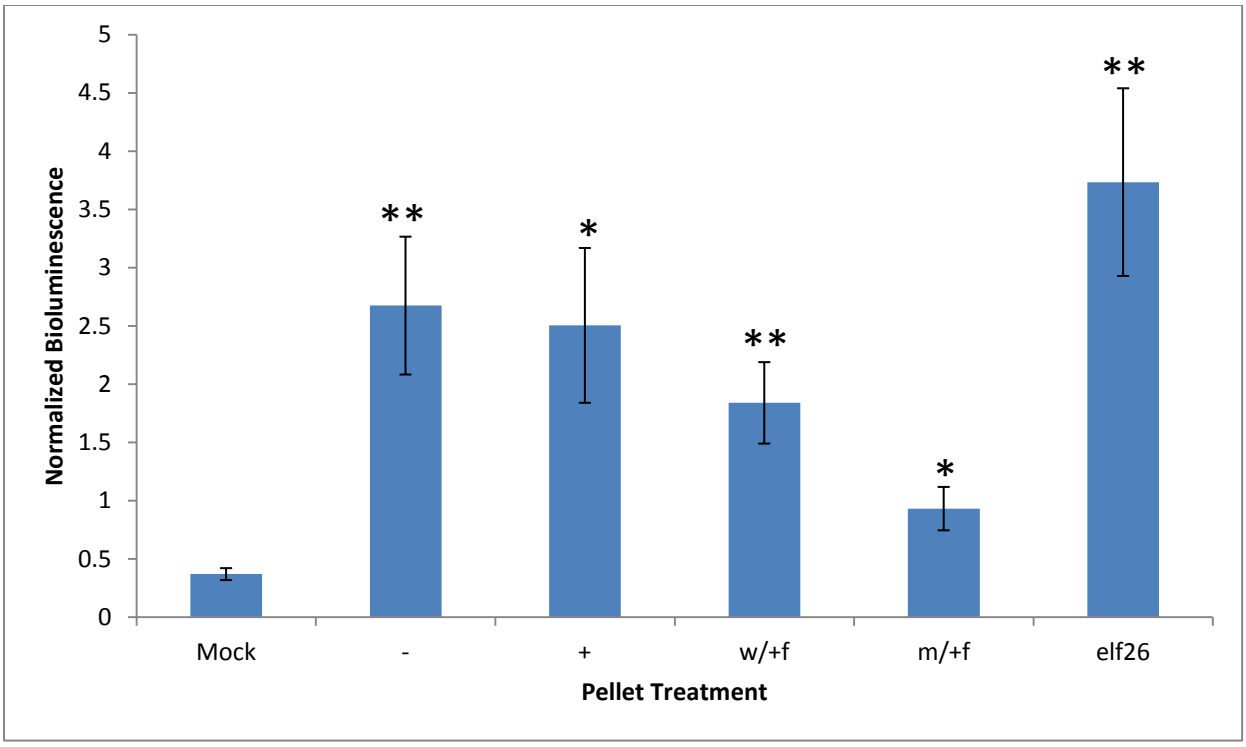

Figure 24 Bioluminescence of aeuqorin-coelenterazine complex in response to calcium influx initiated during a MAMP response of *A. thaliana* treated with mock treatment, elf26, or pelleted *P. syringae* pretreated with –f, +f, w/+f, or m/+f. Error bars are standard error. ($p < 0.05$ is denoted by *; $p < 0.01$ denoted by ** with respect to mock treatment)

Figure 25 exhibits results from treating *A. thaliana* with the supernatant in which *P. syringae* were cultured. Application of the mock treatment to *A. thaliana* seedlings elicited low bioluminescence, which served as the negative control for this experiment. *A. thaliana* treated with the MAMP elf26 served as the positive control and elicited bioluminescence significantly higher ($p < 0.05$) than the negative control (Figure 25). *A. thaliana* treated with *P. syringae* pretreated with –f bioluminesced with levels that closely resembled bioluminescence from the control and therefore were not significant. *A. thaliana* seedlings treated with *P. syringae* pretreated with both +f and w/+f bioluminesced at similar levels. However, neither of these treatments yielded any significant difference from the control (Figure 25). Application of *P. syringae* pretreated with m/+f to *A. thaliana* yielded significantly higher levels of bioluminescence ($p < 0.05$) in contrast to the control.

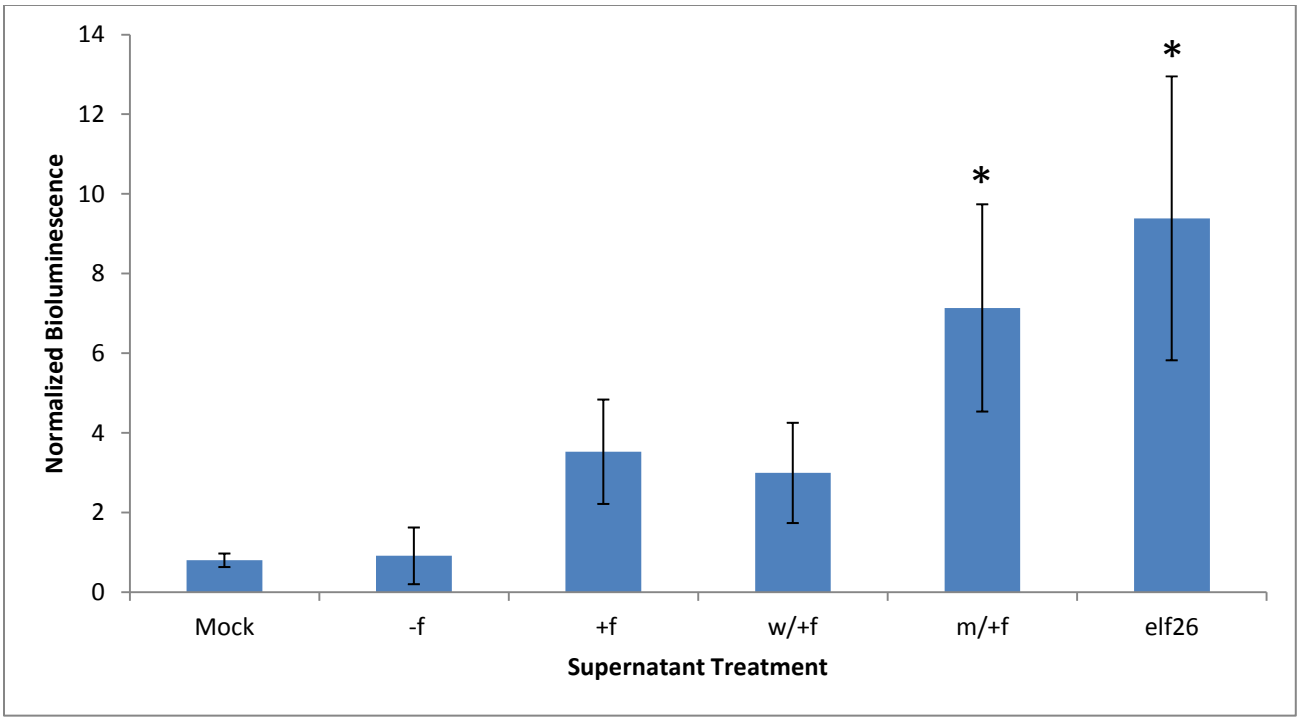

Figure 25 Bioluminescence of aeuqorin-coelenterazine complex in response to calcium influx initiated during a MAMP response of A. thaliana treated with mock treatment, elf26, or supernatant of P. syringae pretreated with –f, +f, w/+f, or m/+f. Error bars are standard

Discussion

Contrasting MAMP Responses Suggest MAMP Dichotomy

ROS and aequorin-calcium assays were used to examine the MAMP response of *A. thaliana* to *P. syringae*. Observed MAMP responses to pelleted *P. syringae* followed the expected bell-shaped curve typical of isolated MAMPs (Aslam et al., 2009) in the ROS assay (Figure 21). Since Aslam and colleagues (2009) demonstrated that addition of multiple MAMPs interact synergistically, it would seem plausible that *P. syringae*, containing multiple MAMPs, would also elicit MAMP responses higher than that of elf26 alone. However, elf26 elicits a higher MAMP response because of its purity, size, and mobility (Aslam et al., 2009). Peculiarly, calcium-aequorin assays did not demonstrate similar potency between experiments. In fact, ROS production was inversely proportional to calcium influx (Figure 21 and 24, Table 1).

Observed MAMP responses to the supernatant of *P. syringae* demonstrated the characteristic bell-shaped curve typical of isolated MAMPs (Aslam et al., 2009) (Figure 22). Unlike MAMP response elicited by pelleted *P. syringae*, the supernatant elicited a more gradual prolonged curve (Figure 22). MAMP responses of *A. thaliana* treated with *P. syringae* pretreated with w/+f and m/+f were stronger than the isolated elf26 (Figure 22 and Table 2), which indicates a high concentration of detectable MAMPs. Calcium-aequorin assays echoed the potency of MAMP responses to pelleted *P. syringae*, demonstrating that in both assays ROS production and calcium fluctuated similarly in each treatment (Figure 24).

There are three main differences between MAMP response elicited by pelleted *P. syringae* and supernatant of *P. syringae*. First, pelleted *P. syringae* elicited inversely proportional MAMP responses with respect to calcium influx and ROS production (Figure 21 and 24), while supernatant of *P. syringae* elicited directly proportional MAMP response with respect to calcium influx and ROS production (Figure 22 and 25). Second, the supernatant of *P. syringae* demonstrated a gradual prolonged curve characteristic of weaker MAMP response (Figure 22). Third, elicitation by supernatant of *P. syringae* pretreated with w/+f and m/+f demonstrated higher MAMP response than even purified elf26 (Figure 22).

Together these differences suggest that MAMPs secreted into the supernatant and MAMPs anchored in bacterium elicit varied responses that possibly possess different immune functions. Thus, two fundamentally different types of MAMPs are proposed: secreted MAMPs (sMAMPs) and anchored MAMPs (aMAMPs). This necessarily means that for sMAMPs and aMAMPs there are respective secreted MAMP PRRs (sPRRs) and anchored MAMP PRRs (aPRRs). These are only classifications and do not connote new functions to already characterized MAMP responses. For example, perception of flg22 and elf26 do not elicit identical responses, but their ultimate purpose is to activate WRKY transcriptions

factors that modulate the immune response. The underlying nature of this dichotomy, and particularly that of aMAMPs, will be explored and developed in subsequent sections.

In summary, MAMPs are able to be divided into two distinct types of MAMPs: aMAMPs, which remain fastened to bacterial cells walls and plasma membrane; and sMAMPs, which are secreted into the extracellular environment. aMAMPs and sMAMPs may contain functions unique to their classification.

Bacterial MAMP Modulation

To determine whether *P. syringae* alters MAMP expression based on past encounters with *A. thaliana*, *A. thaliana* were treated with *P. syringae* pretreated with *A. thaliana* exudates. *P. syringae* pretreated with either –f or +f elicited a variety of MAMP responses from *A. thaliana*. The ROS assay indicated that *A. thaliana* treated with the pellet or supernatant of *P. syringae* pretreated with either –f or +f demonstrated the weakest MAMP response (Figure 21, 22, and 25). *A. thaliana* treated with pellet or supernatant of *P. syringae* pretreated with w/+f or m/+f elicited stronger MAMP responses than those pretreated with –f or +f (Figure 21 and 22). Between w/+f and m/+f pretreated *P. syringae*, *A. thaliana* treated with *P. syringae* pretreated with m/+f elicited the strongest MAMP response (Figure 21 and 2).

This suggests that *P. syringae* that have previously contacted *A. thaliana* elicit a stronger response than those without previous contact. After initially contacting *A. thaliana, P. syringae* may prepare for infection either by upregulating MAMP expression or deactivating a mechanism that disguises bacteria from plants when they are not infecting. Production of MAMPs by bacteria in response to encountering plants is not only possible, it is likely. In legumes, nitrogen fixing bacteria and plants communicate through molecular and chemical interchanges, which cause upregulation and down

regulation of proteins where necessary to coexist in a mutualistic relationship (Wang et al., 2012). However, new MAMP production is not the only possibility for increased MAMP responses as a result of exposure to plants. It is understood that bacteria are capable of disguising their presence via suppression of the host immune response during infection (Jones and Dangl, 2006), so it seems plausible they may also contain a mechanism that allows them to disguise their presence before infection as well. Bacteria could use such a mechanism to remain hidden until their numbers reach a certain threshold and are capable of infection.

Previously unpublished data from Anderson and Peck (2011) have demonstrated that *P. syringae pv.* DC3000 containing a *LuxCDABE* gene bioluminesce in the presence of *A. thaliana* (Supplementary Figure S6) and secreted a biofilm. Both production of the luciferase protein from the *LuxCDABE* gene and secretion of biolfilm are characteristic of bacteria that utilize quorum sensing. Quorum sensing (QS) is used by bacteria to monitor colony populations and to communicate with one another via secreted proteins and chemicals (for review see Li and Tian, 2012). Combining unpublished findings from Anderson and Peck (2011) with QS, it seems plausible that bacteria might use QS to communicate not only about whether nutrients are available or their population is reaching carrying capacity, but also whether there are enough bacteria present to risk infection of a host. If bacteria are preparing to infect a host, it is strategically advantageous to wait until their numbers are capable of overcoming the immune system. If they invade too early, there is a high probability the immune system will incapacitate and overwhelm the pathogens. To do this, bacteria would need to molecularly identify hosts. Though not a foreign concept, bacteria are capable of using external biotic and abiotic stimuli to alter their behavior. Venturi and colleagues (2011) review how external stimuli signal to and activate the QS system of *Pseudomonas*. Before the conception and definition of QS in bacteria, it was known that the bacterium *Erwina carotovora* was able to receive signals as secreted molecules from other bacteria (Pirhonen et al., 1993; Jones et al., 1993). Thus, it seems plausible that bacteria could also

receive signaling molecules from plants. In fact, Polluma and colleagues (2012) discuss the role of QS in the production of virulence factors such as the plant cell-wall degrading enzymes secreted by Pectobacterium carotovorum and *Pectobacterium atrosepticum*. In their review, Polluma and colleagues (2012) provide examples of involvement of QS in expressing virulence factors. Thus, it is possible that *P. syringae*, like *P. carovorum* and *P. atrosepticum*, may be able to modulate expression of, and therefore, disguise their MAMPs until prepared for the infection process.

Production of and un-concealment of MAMPs may be synonymous. Despite being separate reasons for increased MAMP response strength, one does not necessarily preclude the other. In bacteria, producing fewer MAMPs than are detectable (production) may provide the same result as producing proteins or chemicals that conceal MAMPs (concealment). Between the two, increased MAMP production is more plausible as it would likely require less energy to produce a set of MAMPs via QS than to constitutively produce concealment proteins. However, since MAMPs are generally required for survival in pathogens, total concealment is impossible as seen by *A. thaliana* treated with *P. syringae* pretreated with –f or +f (Figure 21).

To recapitulate the main points, *P. syringae* that previously encountered *A. thaliana* exudate elicited higher MAMP responses in *A. thaliana* than those treated with either –f or +f. Increased MAMP responses to *P. syringae* pretreated with *A. thaliana* exudate are thought to result from either an ability to disguise its MAMPs or produce new MAMPs. However, MAMP production seems more likely because it would likely cost less energy. Regardless, QS is implicated in the process by which *P. syringae* suppress a MAMP response in *A. thaliana*.

Bacterial Interaction with *A. thaliana* MPK4 Knockouts

MPK4 is an inhibitor that prevents constitutive activation of the immune response (Figure 13). *A. thaliana* with mutant *mpk4* exhibit increased pathogen resistance and a dwarfed phenotype (Nakagami et al., 2006; Gao et al., 2008). Under natural conditions, if *P. syringae* encountered *A. thaliana* with *mpk4* mutations, the success of infection would be drastically reduced because *A. thaliana*'s immune response would be constitutively activated and thwart attempts to block the immune response. To test whether *P. syringae* would differentially alter MAMP expression in response to molecular differences within *A. thaliana*, *P. syringae* were exposed to mutant *A. thaliana* with *mpk4* knockouts (*mpk4* -/-) and used to treat col-0. *A. thaliana* treated with *P. syringae* pretreated with m/+f demonstrated the highest MAMP responses in figures 21, 22, and 25 and the weakest response in figure 24.

Comparisons of stronger MAMP responses in *A. thaliana* elicited by the treatment of *P. syringae* pretreated with m/+f to weaker MAMP responses elicited by the treatment of *P. syringae* pretreated with w/+f indicate that *P. syringae* can alter MAMP expression or concealment based on molecular differences in plants (Figures 21, 22 and 25). How *P. syringae* recognizes the absences of MPK4 is not understood since MPK4 is not available for direct detection. One possibility is that bacteria are capable of detecting the increased concentrations of ROS or SA that typically accompany *mpk4* mutants (Broderson et al., 2006; Nakagami et al., 2006; Gao et al., 2008). Alternatively, bacteria may simply recognize that their infection efforts are futile and as a result increase MAMP expression or un-concealment. In Figure 24, *P. syringae* pretreated with m/+f elicted the weakest MAMP response. Since results from Figure 24 inversely contrast all previous data, these data will be discussed separately.

To sum up these results, *P. syringae* was able to recognize constitutive activation of the *A. thaliana* immune response and accordingly adjust its MAMP expression. Generally, this differential

MAMP expression resulted in stronger MAMP responses (Figure 21, 22, and 25), but in one instance resulted in a weaker MAMP response (Figure 24).

A Negative Feedback System Suppresses MAMP Responses

In contrast to results obtained from the ROS assay, the aequorin-calcium assay indicates that MAMP responses of *A. thaliana* treated with *P. syringae* are more complicated and less straightforward than a simple ROS assay can detail (Figure 24 and 25). *A. thaliana* treated with pelleted *P. syringae* pretreated with –f and +f elicit a calcium influx on par with elf26 (Figure 24). *A. thaliana* treated with *P. syringae* pretreated with w/+f and m/+f are significantly greater than the mock, but do not elicit a calcium influx nearly as strong as that caused by *A. thaliana* treated with *P. syringae* pretreated with w/+f. These results are difficult to interpret as they seem to contradict findings that calcium influx is greater in *A. thaliana* treated with supernatant of *P. syringae* pretreated *A. thaliana* exudate and less when treated with –f or +f (Figure 25). If taken at face value, these results seem to imply that pelleted *P. syringae* employ MAMP expression nearly opposite those suggested by results from the ROS assay (Figure 21). However, Figure 25 indicates that MAMP responses are similar to those described by ROS assays. Figure 25 displays an increase in MAMP response with respect to bacterial pretreatment. *P. syringae* pretreated with –f demonstrated the lowest MAMP response, while those pretreated with m/+ demonstrated the highest MAMP response next to elf26.

The apparent discrepancy in these data can be explained by a negative feedback system that is dependent upon the MAMP perceived by *A. thaliana*. First, it is possible that *A. thaliana* can endocytose PRRs of aMAMPs. It has already been demonstrated that plants are capable of endocytosing the sMAMP receptor FLS2 when high concentrations of flg22 are present (Robatzek et al., 2006). This

endocytosis effectively ends the MAMP response. Second, it is possible that an intermediate protein interacts with either the PRR or NADPH oxidase. Robatzek and colleagues (2006) proposed that a kinase-associated protein phophatase (KAPP) could interact with the receptor-like kinase (RLK) FLS2.

The mechanisms for these proposed negative feedback loops begin with aMAMP perception and calcium influx. If the negative feedback loop is controlled by endocytosis of PRRs, it is likely that inhibition of ROS production occurs because PRRs are separated from the membrane-bound NADPH oxidases and therefore cannot trigger their activation.

Alternatively, an intermediate protein could be activated at high $[Ca2+]_{cyt}$ and either dephosphorylate PRRs of aMAMPs or interact in an inhibitive manner with NADPH oxidase. Either would inhibit ROS production, resulting in the inverse relationship between Ca2+ influx and ROS production (Figure 21 and 24). For this mechanism to exist exclusively for aMAMP perception, as suggested by these data, the intermediate protein would have to associate with aPRRs but not with sPRRs. Since aPRRs have yet to be isolated and characterized, it seems plausible that binding of an aMAMP to this receptor could activate a negative feedback loop either through proteolytic cleavage of the cytosolic domain or phosphorylation of an intermediate protein.

Regardless, it is expected that activation results in the exposure of an EF-hand motif that can bind Ca^{2+}. Binding of Ca^{2+} allows this protein to either dephosporylate aPRRs or bind NADPH oxidases to inhibit production of ROS. For this reason, when high levels of MAMPs are detectable as is the case with *P. syringae* pretreated with –f, a high concentration of Ca^{2+} enters the cell (Figure 24: –f) and activates NADPH oxidases, producing ROS. ROS production is subsequently inhibited by the simultaneous activation of a high number of negative feedback loop proteins. This results in lower concentrations of ROS being produced (Figure 21: –f). By contrast, when fewer MAMPs are detectable as is the case with

m/+f, less Ca^{2+} enters the cell (Figure 24: m/+f). Lower concentrations of Ca^{2+} result in activation of fewer inhibitory negative feedback loop proteins

Interestingly, this particular negative feed loop does not appear to exist in the detection of sMAMPs. Upon detection of sMAMPs, there is a Ca^{2+} influx. As Ca^{2+} increases (Figure 25), so too does the production of ROS (Figure 22). As seen in figure 22, *P. syringae* pretreated with –f elicited the weakest MAMP response and *P. syringae* pretreated with m/+ elicited the greatest MAMP response.

Together, these data suggest a MAMP response dichotomy to match the earlier proposed MAMP dichotomy. First, it appears that detection of aMAMPs possess a negative feedback loop. Second, detection of sMAMPs lacks this particular negative feedback loop. This is understandable because sMAMPs directly contribute to virulence. Removal of these MAMPs result in impotence. By contrast, anchored MAMPs are indirectly involved in virulence. Removal of these MAMPs also results in impotence, but only because it typically kills the cell. In other words, detection of sMAMPs is characteristic of an invading pathogen, while aMAMPs could indicate either the invasion of a pathogen or the attempted union of a mutualistic organism. Bacteria are capable of living in many locations and not all bacteria are pathogens. Constitutively activated *PR* genes in response to non-pathogenic bacteria utilize valuable resources and hinder plant growth. Thus, it would be beneficial for plants to possess a negative feedback system to suppress a MAMP response to non-pathogenic bacteria.

These inferences do not undermine the concept that bacteria regulate MAMP expression or concealment nor do they affect the results obtained from the *mpk4* -/- experiment. It only adds another layer of complexity to an already complex system. However, understanding how bacteria and plants naturally modulate protein expression with respect to one another is more important than feigning ignorance to the enumerable mechanisms that complicate systems.

In summary, aMAMPs contain a negative feedback loop that explains the inverse relationship between Ca2+ influx and ROS production. sMAMPs lack this negative feedback system. The feedback system likely exists to prevent over-allocation of resources toward fighting a presumed infection when in actuality detection of high concentrations of aMAMPs is more likely to indicate the death of many bacteria since aMAMPs are fastened to the cell wall and plasma membrane.

Experimental Critique

Though many interesting proposals as to how *P. syringae* affect the MAMP response in *A. thaliana* have been discussed, there are possible flaws in the logic used to describe these data. First, it is important to note that *A. thaliana* of two different ages were used for this experiment. Plants of 4 – 5 weeks in age were used for ROS assays and 2 – 3 day old seedlings were used for aequroin-calcium assays. In this experiment, the assumption was made that there is no difference between the MAMP response systems in seedlings and adult plants. However, this is not true. There have been concerns indicating the MAMP responses function differently in seedlings as opposed to adult plants (Peck, unpublished data).

Additionally, different parts of plants were examined during this experiment. The ROS assay utilized discs removed from leaves while the aequorin-calcium assay utilized the entire seedling. Once again, the assumption was made that different portions of *A. thaliana* contain the same MAMP response system. Findings from Robatzek and colleagues (2006), demonstrated that MAMP responses in roots differ considerably from those in leaves. Once again the assumption of equality has been dispelled. Unfortunately, this truly only makes the ROS assay results relatable to the leaves of adult plants between the ages of 4 – 5 weeks and the aequorin-calcium assay results applicative to 2 – 3 day old seedlings. Though it is expected that similar results would be obtained if one respectively performed

the ROS or calcium-aequorin assay in seedlings and adult plant leaves, this will not be concluded until further experimentation demonstrates otherwise.

Additionally, experimental resolution poses difficulty in interpreting these data. ROS assays provide information about ROS generation and aequorin-calcium assays provide information about calcium influx, but neither is a direct method of measuring the amount of MAMPs present. Generation of ROS or influx of calcium indicate that an external signal was received by *A. thaliana* and that respectively NADH oxidases have been activated or calcium channels have opened. However, as mentioned, a wide array of external stimuli result in ROS production. Similarly, many external stimuli also trigger Ca^{2+} influx. Though a tentative MAMP response can be examined using ROS and aequrin-calcium assays, the only information that can be gleaned from these data are that a MAMP response occurred and that it was relatively strong or weak in comparison to other MAMP responses in the trial. Since all MAMP responses are measured in relative light units, data sets cannot be compared to another without applying a method for normalizing the data.

ROS assays are conducted under the assumption that plants are the only producers of ROS. However, bacteria produce ROS via proteins like fumarate reductase and sulfite reductase (Messner and Imlay, 1999; Meehan and Malamy, 2012). Recent findings have shown that the ROS H_2O_2 generated in a genetically synthetic bacterium is capable of sending messages to other nearby synthetic bacteria (Prindle et al., 2012), suggesting that ROS could exist as a form other than a byproduct. Though *P. syringae* are dead, it is not feasible to assume that *A. thaliana* is the only source of ROS.

In addition to being unable to determine the concentration of MAMPs, the quality of MAMP response to pathogenic MAMPs cannot be determined. Many isolated MAMP peptides, such as elf26 or flg22, are ideal elicitors. Aslam and colleagues (2009) noted that truncated versions of MAMPs are likely more potent than those that naturally occur. This deviation from natural potency is the result of

altering a MAMP's structure. Similarly, in nature, rarely do proteins share one hundred percent consensus with homologs in other species. MAMPs may contain different amino acid sequences, post-translational modifications, and/or transcriptional or expressional levels from one pathogen to another. Such modifications can alter a plant's perception of a particular MAMP. As such, not all bacterial MAMPs may share perfect sequence consensus and be identified equally.

Also, different bacteria possess varying degrees of virulence, which can be partially consigned to their MAMP expression levels. Though it is traditionally thought that increased virulence of a pathogen will lead to a greater MAMP response, this is not definite. Whether a bacterium is identified by the plant immune system and elicits a MAMP response is contingent upon whether bacterial MAMPs or effectors are recognized (Jones and Dangl, 2006). Failure to recognize MAMPs or effectors of even the most virulent bacterium will result in the elicitation of a weak MAMP response. Table 3 is a qualitative representation of the importance of MAMP perception. If MAMPs are not perceived there can be no immune response.

	Virulent	Not Virulent
High MAMP Perception	High	High
Low MAMP Perception	Low	Low

Table 3 Qualitative representation of MAMP perception. Note that virulence has little to do with whether a MAMP response is elicited, but rather MAMP perception is the key factor.

Thus, the resolution with which these experiments were conducted cannot differentiate MAMP concentration from MAMP perception. It is just as likely that new MAMPs were produced as it is that preexisting MAMPs were un-concealed.

For these reasons, these findings should be read with scrupulous eyes and used as a basis from which other detailed experiments can be performed to control for such variables and prove or disprove these findings.

Future Directions

This experiment was a preliminary study conducted to determine whether bacteria are capable of modulating MAMP expression. However, after examining the data, it is obvious that certain assumptions, lack of resolution, and unexpected results contributed to uncertainty in certain portions of the data. In future experiments, results could be improved by modifying the methods. First, perhaps the most important alteration capable of lending credence to these results is controlling for the age and anatomy of *A. thaliana.* Second, the experiment could be repeated, but instead of allowing bacteria to remain whole, aMAMPs could be liberated through sonication. Additionally, removal of the bacterial cell wall, which contains many of the aMAMPs, and sonication could serve as a negative control to which data can be compared. Third, *P. syringae* could be tested for the ability to 'remember' previously encountered hosts by introducing more time between pretreatment of *P. syringae* and treatment of *A. thaliana*. This would allow time for bacteria to break down or re-conceal any MAMPs during pretreatment. After pretreatment, *P. syringae* could be cultured and then used to treat *A. thaliana*. Variability between *P. syingae* pretreated with plant exudate and those pretreated with –f or +f would suggest that *P. syringae* maintain a 'memory' of hosts they have encountered.

Additionally, an entire series of new experiments could be conducted from the results procured from this experiment. First, it was suggested that bacteria utilize QS to coordinate the expression or concealment of MAMPs. To test the role of QS in bacterial MAMP alteration, a QS inhibition protein called Autoinducer-2 (AI-2) would be added to disrupt bacterial communication during pretreatment of *P. syringae*. Then, similar experiments to those performed here would be executed.

Second, a negative feedback loop was proposed to suppress the MAMP response. To determine the existence of such a mechanism the preexisting method of PRR deactivation would be examined. As

mentioned, FLS2 MAMP sensing is deactivated through endocytosis. To determine whether aPRRs are deactivated in a similar manner, caveolae-mediated endocytossis would be inhibited using Nystatin, Filipin III, and/or Genistein. Similarly, clatherin-mediated endocytosis would also be inhibited with wortmannin. Inhibitions of both types of endocytosis would be separately and concomitantly conducted to determine whether more than one process functions simultaneously to achieve aPPR deactivation.

If endocytosis did not play a role in aPRR deactivation, an *in silco* search for various aPRRs would be undertaken by examining genes that contain high conservation of LRR domains. Additionally, as a side search, sequences that match the binding site of TLR4 would be sought in attempt to isolate the plant PRR for LPS. After identification of possible genes that produce PRRs, a cDNA library would be constructed containing mutant or knockouts PRR genes. *A. thaliana* with deficient immune responses to aMAMPs would be screened. Proteins that caused deficient immune responses in mutants would be co-immunoprecipitated with any bound ligands. Finally, bound ligands would be chararacterized in hopes of elucidating this negative feedback mechanism.

Conclusion

In conclusion, *P. syringae* is capable of altering MAMP detection in response to having previously encountered *A. thaliana*. *P. syringae* also appears to recognize whether the host is wild type A. thaliana (col-0) or mutant mpk4 -/- *A. thaliana*, which suggests that *P. syringae* can gauge the success of its infection. The mechanism that allows *P. syringae* to suppress the MAMP response and 'remember' *A. thaliana* is yet unknown, but is suggested to relate to MAMP expression or MAMP concealment. Additionally, it seems likely that *P. syringae* participate in quorum sensing to achieve this alteration of MAMP detection. Additionally, a MAMP dichotomy was determined and negative feedback loop is implicated in reducing ROS production when *A. thaliana* perceives aMAMPs. Refining current

experimental methods and building from these experiments could yield more conclusive and less inferred results. Regardless, these findings contain promising prospects from which to construct future experiments; the fruitful results of which may yield possible ways to protect crops and biofuels from pathogens.

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3. Figure 2B: http://genome.jgi-psf.org/psesy/psesy.home.html
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6. Figure 9C: http://arabidopsis.info/students/agrobacterium/

7. Figure 15: (http://plastid.msu.edu/images/petri_dish.jpg

8. Figure 16: unbc.ca/efl/equipment.html

9. Figure 17: directindustry.com/prod/photek/light-tight-dark-boxes-34310-224798.html

10. Figure 18:

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13. Figure 20B:

http://www.biotechniques.com/multimedia/archive/00108/BTN_A_000113533_O_F_108580b.jpg

Appendix A: **Acronyms**

AC9 – anthracene-9-carboxylate

AI-2 – autoinducer-2

aMAMP – anchored microbe-associated molecular pattern

aPRR – anchored MAMP PRR

AtEFR – *Arabidopsis thaliana* elongation factor receptor

AtrbohD – *Arabidopsis thaliana* respiratory burst oxidase homolog D

AtrbohF – *Arabidopsis thaliana* respiratory burst oxidase homolog F

ATP – adenosine triphosphate

AvrB – avirulence protein B

AvrRPM1 – avirulence protein (recognized by) RPM1

AvrRpt2 – avirulence protein (recognized by) root photostropism protein 2

Avr proteins – avirulence proteins

BAK1 – BRI-associated kinase 1

BR – brassinosteroid

BRI – brassinosteroid insensitive-1

Ca^{2+} – calcium cation

$[Ca^{2+}]_{cyt}$ – concentration of cytosolic calcium cations

cADPR – cyclic adenosine dipshophate ribose

CaM – calcium modulating protein or calmodulin

Cdc53 – cell division control protein 53

CERK1 – ceramide kinase 1

cGMP – cyclic guanosine monophosphate

Col-0 – Arabidopis *thaliana* ecotype Columbia

DNA – deoxyribonucleic acid

DPI – diphenylene iodonium

Dra-0 – *Arabidopsis thaliana* ecotype Drahonin

EF-Tu – elongation factor thermo unstable

EFR – elongation factor receptor

Elf18 – 18 amino acid peptide from EF-Tu

eLRR – extracellular leucine rich repeat

ERK – extracellular signal-receptor kinase

ERM – elicitor-responsive MAPK

ETI – effector-triggered immunity

ETS – effector-triggered susceptibility

FLS2 – flagellin-sensitive 2

GFP – green fluorescent protein

GPI – glycophosphatidylinositol

$\bullet HO_2$ – hydroperoxyl anion

H_2O_2 – hydrogen peroxide

HR – hypersensitive response

iNOS – inducible nitric oxide synthase

KAPP – kinase-associated protein phosphatase

MAMP – microbe-associated molecular pattern

MAPK – mitogen-activated protein kinase

MEK – MAPK/ERK kinase

MEK2 – MAPK/ERK kinase kinase

MG132 - N-(benzyloxycarbonyl)leucinylleucinylleucinal

Mg^{2+} – magnesium cation

MKS1 – MAP kinase substrate 1

MPK3 – MAP Protein Kinase 4 (orthologs of WIPK)

MPK6 – MAP Protein Kinase 6 (ortholog of SIPK)

NADPH – nicotinamide dinucleotide (reduced)

NB-LRR – nucleotide binding leucine rich repeat

NDR1 – non-race specific disease resistance protein 1

NO – nitric oxide

NOS – nitric oxide synthase

Nrobh – *Nicotiana* respiratory burst oxidase homolog

Ntf4 – *Nicotiana tabacum* MAPK4

$\bullet O_2^-$ – superoxide

$\bullet$OH – hydroxyl radical

PAL – phenylalanine lyase

PAMP – pathogen-associated molecular pattern

Po-0 – *Arabidopis thaliana* ecotype Germany

PR gene – pathogen-resistance gene

PR-1 protein – pathogenesis related-1 protein

PRR – pattern recognition receptor

PTI – pathogen triggered immunity

Pseudomonas syringae pv. DC3000 – Pseudomonas syringae pathovar DC3000

QS – quorum sensing

R genes – resistance genes

R proteins – resistance proteins

Rboh – respiratory burst oxidase homolog

RIN4 – RPM1-interacting protein 4

RLK – receptor-like kinase

ROS – reactive oxygen species

RPM1 – synonym for Resistance to *Pseudmonas syringae* protein 3

RPS2 – Resistance to *Pseudmonas syringae* protein 2

RPS3 – Resistance to *Pseudmonas syringae* protein 3

SA – salicylic acid

SCF – skp1–cdc53cullin–F-box

Skp1 – S-phase kinase-associated protein 1

SIPK – salicylic acid-induced protein kinase

sMAMP – secreted microbe-associate molecular pattern

sPRR – secreted MAMP PRR

T-DNA – transfer deoxyribonucleic acid

VIP1 – VirE2-interacting protein 1

VirE2 – Virulence factor E2

VirF – Virulence factor F

Ws-0 – *Arabidopsis thaliana* ecotype Russia

WIPK – wound-induced protein kinase

WRKY – (Superfamily of plant transcription factor)

Appendix B: Model Organisms

Animals

Aequorea aequorea (A. aequorea)

Bacteria

Agrobacterium tumefaciens (A. tumefaciens)

Erwinia carotovora (E. carotovora)

Helicobacter pylori (H. pylori)

Pectobacterium atrosepticum (P. atrosepticum)

Pectobacterium carotovorum (P. carotovorum)

Pseudomonas aeruginosa (P. aeruginosa)

Pseudomonas syringae (P. syringae)

Pseudomonas syringae pathovar DC3000 (P. syringae pv. DC3000)

Fungi

Phytophthora sojae (P. sojae)

Plants

Arabidopsis thaliana (A. thaliana)

Brassicaceae

Nicotiana benthamiana (N. benthamiana)

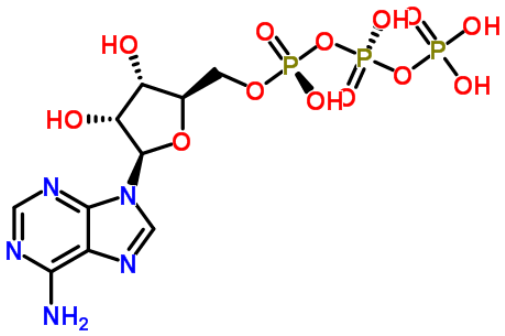
Solanum

Appendix C: Chemical Structures

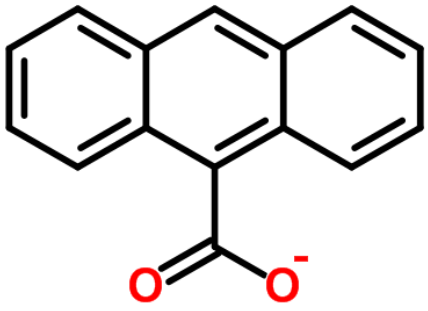
All chemicals that were discussed have their chemical structures presented below. All chemical structures were found using the chemspider search engine at http://www.chemspider.com. Structures are constructed using organic chemistry representations. Carbons exist at the vertices of two or more black lines. The chemical symbols of other elements are used and are color-coded for easy distinction. Hydrogen atoms adopt the color of their parent atom unless they are bonded to carbon in which case they are typically not shown unless for emphasis (as when they play a role in chiral centers). Double and triple bonds are respectively represented by two or three parallel lines between atoms. Solid and

hashed triangular lines respectively indicate bonds formed in front of and behind the central atom to which they are attached. Their purpose is to provide a third dimension to a two dimensional structure.

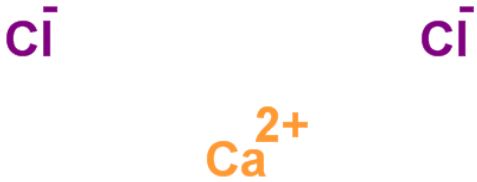
Adenosine triphosphate ($C_{10}H_{16}N_5O_{13}P_3$)



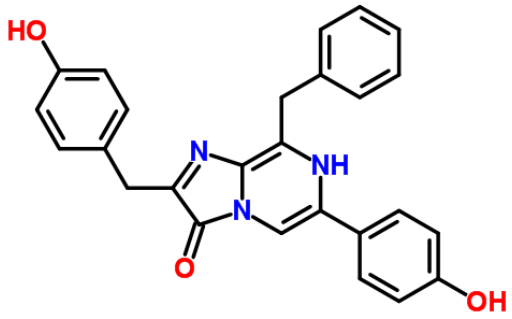
Anthracene-9-carboxylate ($C_{15}H_9O_2$)



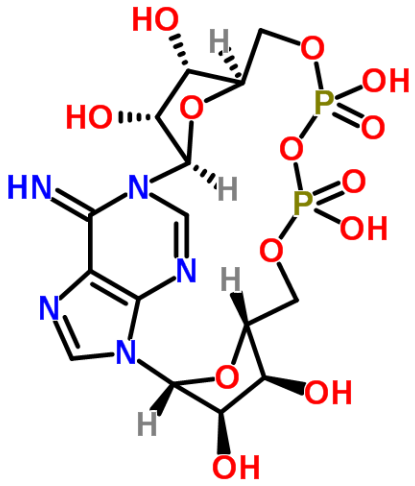
Calcium Chloride ($CaCl_2$)



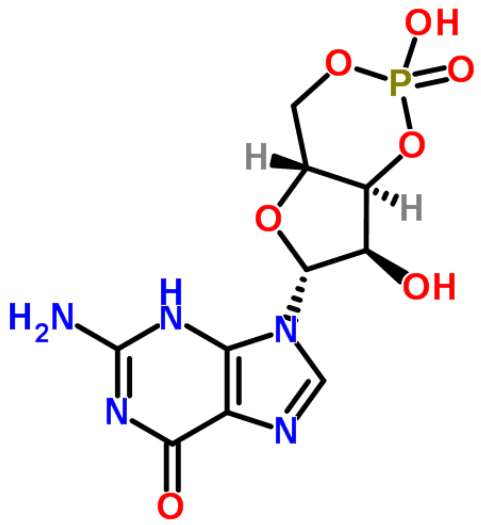
Coelenterazine ($C_{26}H_{21}N_3O_3$)



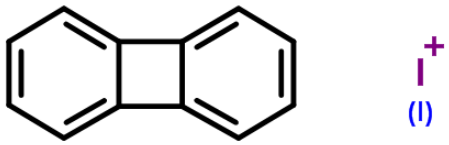
Cyclic adenosine dipshophate ribose ($C_5H_{21}N_5O_{13}P_2$)



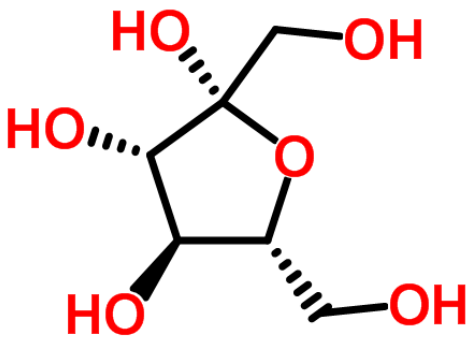
Cyclic guanosine monophosphate ($C_{10}H_{12}N_5O_7P$)



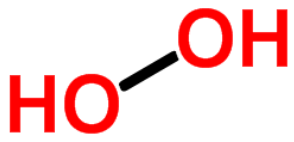
Diphenylene iodonium ($C_{12}H_{10}I$)



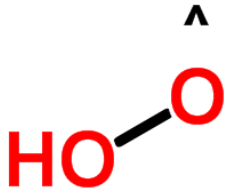
Fructose ($C_6H_{12}O_6$)



Hydrogen peroxide (H_2O_2)

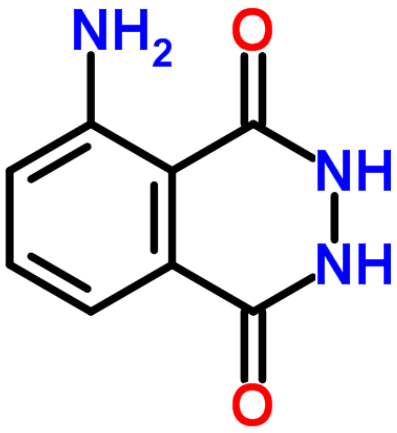


Hydroperoxyl anion ($\bullet HO_2$)

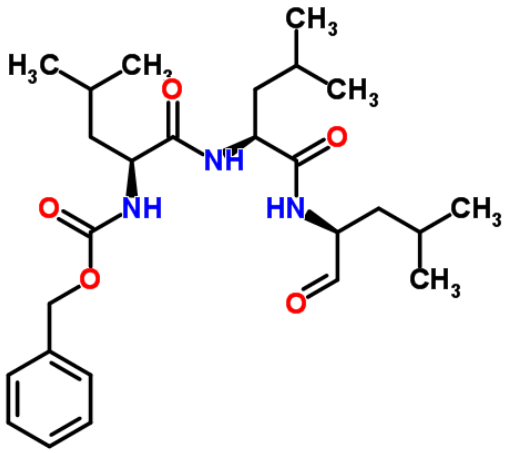


Hydroxyl radical ($\bullet OH$)

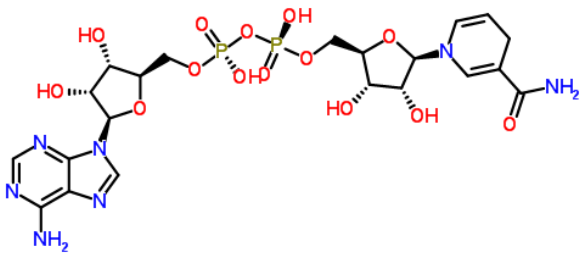
Luminol ($C_8H_7N_3O_2$)



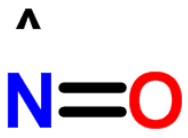
MG132 ($C_{26}H_{41}N_3O_5$)



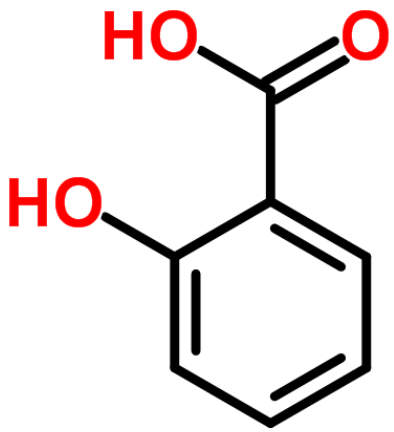
Nicotinamide dinucleotide [reduced] (C21H29N7O14P2)



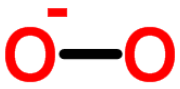
Nitric oxide (•NO)



Salicylic acid ($C_7H_6O_3$)



Superoxide (O_2^-)



Appendix D: Supplementary Review Material

Phosphorylation of Mitogen-activated Protein Kinases

Phosphorylation is a chemical reaction that occurs in three steps. First, the high energy molecule, whose phosphate will be transferred, is brought into proximity of the active site. A nearby amino acid or amino acids will stabilize the negative charge of the two unreactive phosphates (Supplementary Figure S1 Initial state). Magnesium (Mg^{2+}) stabilizes the negative charge of the reactive phosphate (Supplementary Figure S1 Initial state). Second, during the transition state oxygen, or another electronegative atom, begins forming a bond to the phosphate (Supplementary Figure S1 Transition state). Finally, when the bond to phosphate is formed, the bond between the reactive phosphate and unreactive phosphate is broken. All stabilizing Mg^{2+} cations and amino acids release the unreacted phosphates, allowing free dissociation of the product (Supplementary Figure S1 End State).

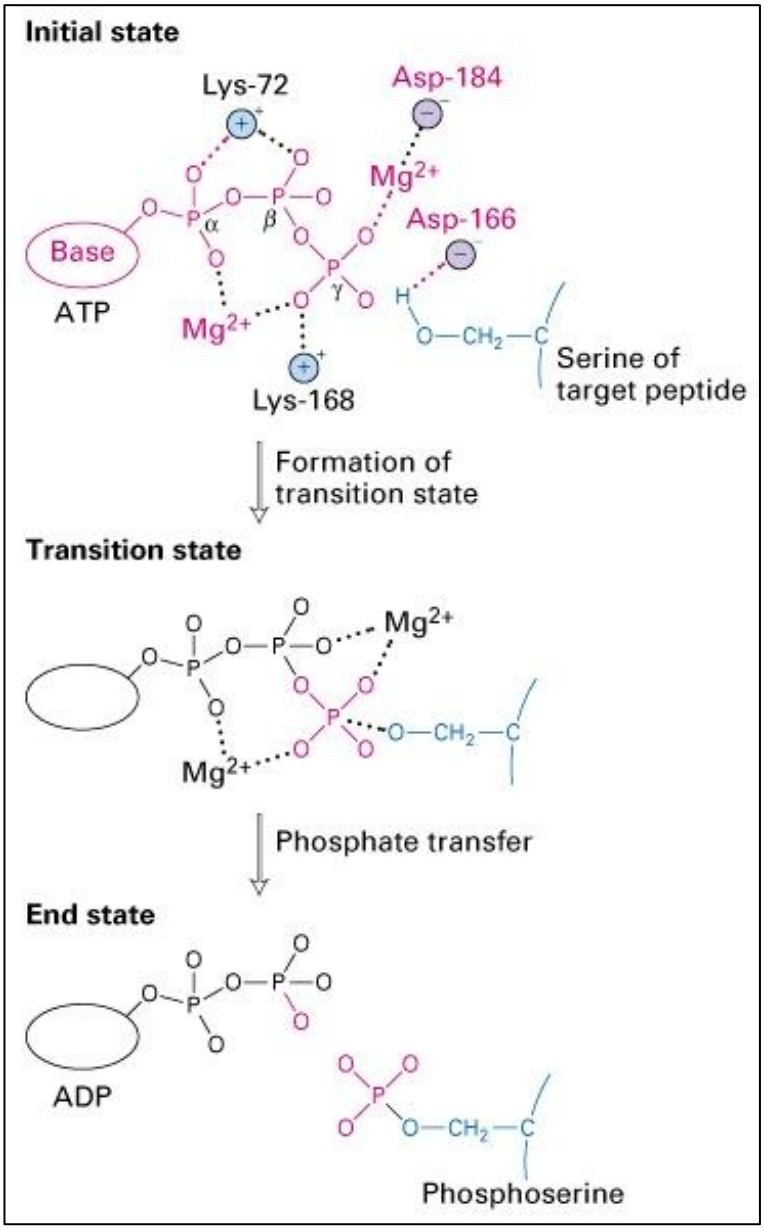


Figure S1 In the initial state ATP is stabilized by Mg^{2+}, Asparagine and Lysine. This helps balance out the oscillating negative charge of ATP. Aparagine accepts as hydrogen from the hydroxyl group of serine (or threonine), which allows the phosphate to make a bond with serine in the transition state. In the end state, ADP and phosphoserine (or phosphothreonine) are the final products, which results in a phosphorylated protein. (adapted from Lodish et al., 2000)

Appendix E: Experimental Reaction Premises

Chemiluminescence of Reactive Oxygen Species Assay

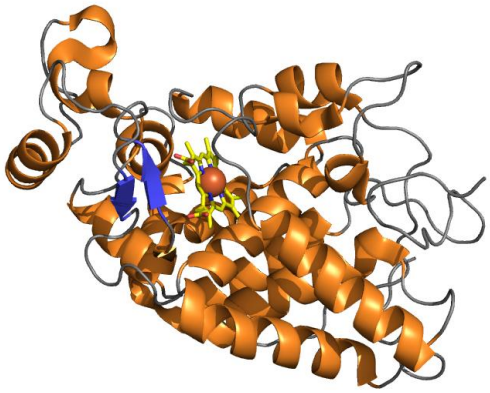


Figure S2 Crystal structure of Horseradish peroxidase coupled with iron heme cofactor (yellow) at resolution of 1.6Å. (adapted from Carlson et al., 2005; protein data base: 1W4Y)

The elicitation of MAMP responses was observed using luminol and horseradish peroxidase (HRP). The elicitation of MAMP responses triggers ROS production (Torres, Jones, and Dangl, 2006; Aslam et al., 2009). Horseradish peroxidase (Supplementary Figure S2) binds these ROS and catalyzes the reduction of luminol to 3-aminophthalate (Supplementary Figure S3) using ROS as the electron donor. Upon completion of this reducton, 3-aminophthalate undergoes an excited triplet state, to an excited singlet state, to a ground state, which concludes with the emission of a photon (*hv*) at 428nm (Supplementary Figure S3). Bioluminescence would occur without the addition HRP as HRP only serves as a catalyst in this reaction.

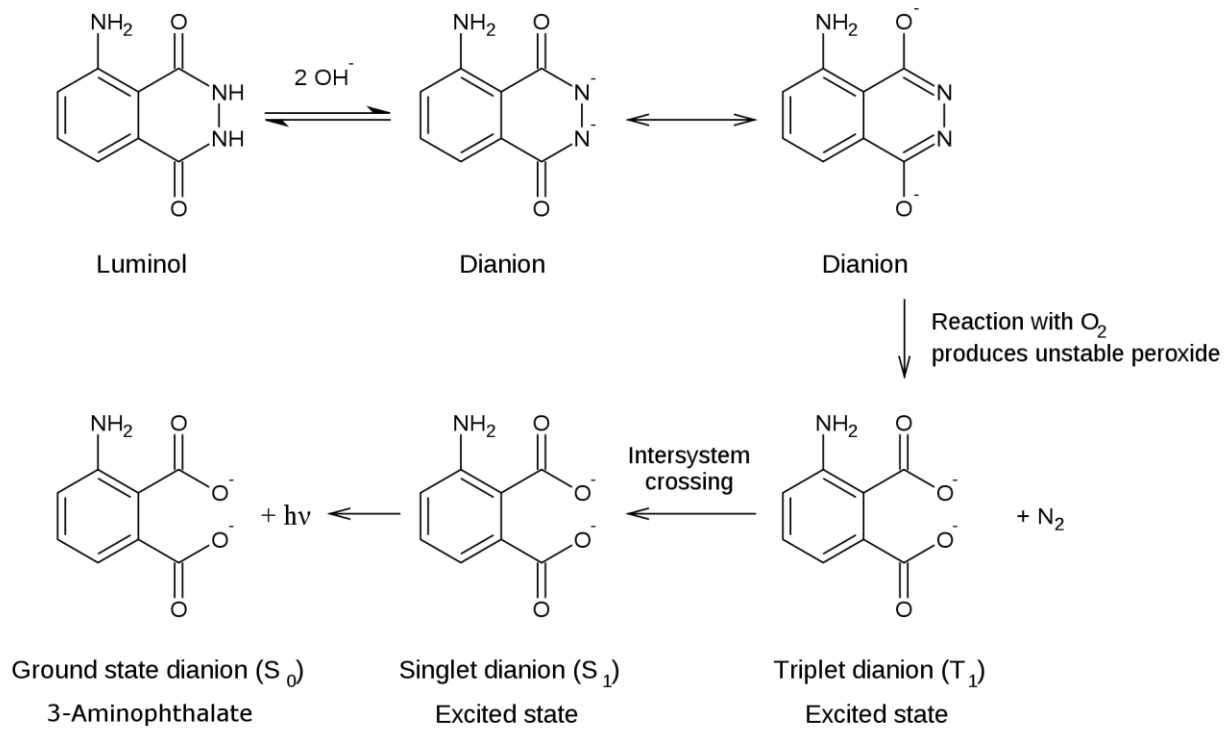


Figure S3 A diagram of the chemical reaction luminol undergoes. **A)** Luminol readily reacts with ROS to form a dianion. **B)** The dianion undergoes is converted to a resonance structure. **C)** The resonance structure reacts with molecular oxygen, producing **D)** an excited triplet state dianion (T1) and Nitrogen gas. The triplet state noradiatively transition to **E)** an excited singlet state (S1) dianion. The singlet state transitions to **F)** the ground state (S0) producing 3-aminophthalate and radiation of a photon. (Adapted from wikipedia user: Fvasconcellos)

Bioluminescence of Aequorin-Calcium Assay

Transgenic *Arabidopsis thaliana* containing aequorin with reconstituted coelenterazine were used to measure the influx of Ca^{2+} during the MAMP response. Aequorin (Supplementary Figure S4) is a protein native to *Aequorea aequorea*, a type of bioluminescent jelly fish. Aequorin contains two units: the apoaequorin unit, which is a gene product; and coelenterazine, which is a cofactor. *In vivo* apoaequorin readily binds coelenterazine to

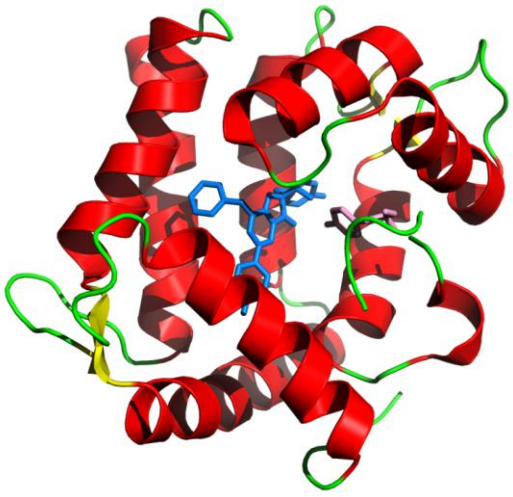


Figure S4 Crystal structure of aequorin photoprotein binding its cofactor coelenterazine (blue) at 2.3 Å**.** (adapted from Head et al., 2005; protein data base: 1EJ3)

produce the functional aequorin product. This product contains four EF-hand motifs. Three of which can bind Ca^{2+} (Head et al., 2000). However, in transgenic *A. thaliana*, coelenterazine cannot be

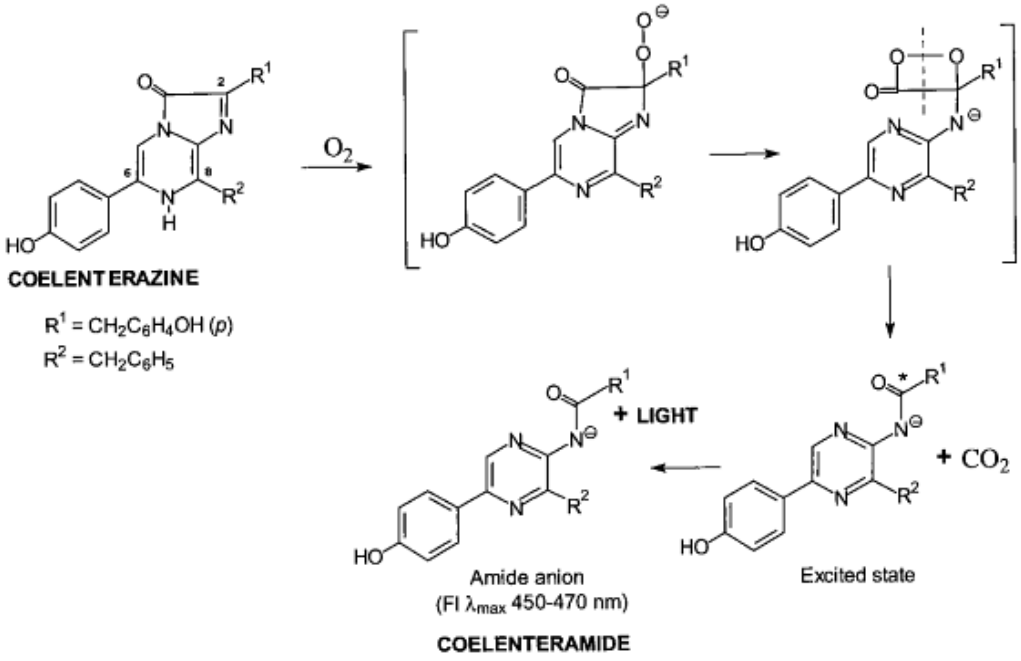


Figure S5 Reaction mechanism between Ca2+-activated apoaequorin and coelenterzine. **A)** Coelenterazine undergoes peroxidation in the hydrophobic binding pocket of aequorin. **B)** The electronegative oxygen of peroxidized coelenterazine transition state attacks the nearby carboxy group, which results in the formation of a **C)** tetricylic group attached to the nitrogen. The tetracyclic group is quickly broken to form **D)** an excited state of coelenteramide and carbon dioxide. The excited state radioactively transitions to **E)** the ground state coelenteramide anion, emitting a photon. (adapted from Shimomura, 2006)

produced because *A. thaliana* lacks the necessary protein machinery to construct the cofactor. This prevents oxidation of coelenterazine despite Ca^{2+} being present. However, due to its hydrophobicity, coelenterazine easily passes through the plasma membrane of *A. thaliana*, and once inside, will readily bind apoaequorin, allowing for the reconstitution of aequorin. Then, any fluctuations in the Ca^{2+} concentration can be measured by bioluminescence generated from the oxidation of coelenterazine (Supplementary Figure S5).

Oxidation of coelenterazine by aequorin occurs when the three EF-hands bind Ca^{2+}. It has been suggested that coelenterazine (Supplementary Figure S5A) is peroxidized by the catalytic triad Tyr184-His169-Try173 (Supplementary Figure S5B) (Head et al., 2000). The negative charge of the oxygen anion attacks the nearest carboxyl group to form an intermediary tetracyclic group (Supplementary Figure S5C). The tetracyclic group is quickly broken, likely as a result of the bonding angles, and an excited state of coelenteramide formed at the expense of forming carbon dioxide (Supplementary Figure S5D). The excited state quickly returns to the ground state emitting a photon of 466nm (Supplementary Figure S5E) (Shimomura, 2006).

Bioluminescence of Pseudomonas syringae pv. DC3000 with LuxCDABE Gene

Plant exudates were tested for their quality by exposing 500µL of plant exudate to 100µL *P. syringae pv. DC3000*. Exposure results in bioluminescence from *P. syringae pv. DC3000* containing the *LuxCDABE* gene. This gene produces a luciferase protein that is transcribed during QS in *Vibrio fischeri* (Miller et al., 2001). Though *P. syringae* normally do not contain the *LuxCDABE* gene, it is still controlled by the promoter for the *LuxI* gene (Supplementary Figure S6), which is also involved in QS. Though *P. syringae* may not contain the identical protein, all Gram-negative bacteria contain a *LuxI*-like gene that is involved in QS (Li and Tian, 2012).

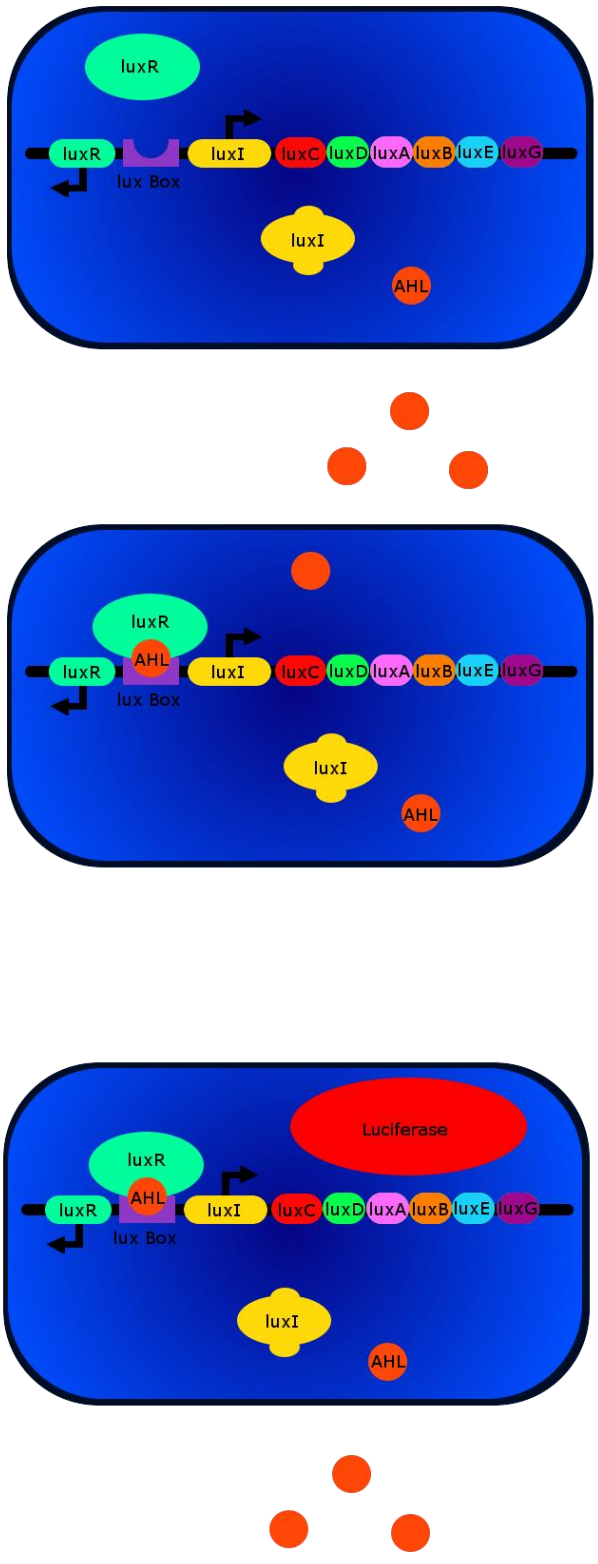


Figure S6 Schematic of QS resulting in transcription of *LuxI* gene, terminating in transcription and translation of *LuxCDABE* gene to a luciferase.

QS begins when *LuxI* is translated into a LuxI protein, which creates acyl-homoserine lactones (AHL), which diffuses across the plasma membrane (Supplementary Figure S6A). AHL diffuses into bacteria within close proximity and binds the LuxR protein translated from the *LuxR* gene. Upon binding, the LuxR-AHL complex binds the lux box, which stimulates further production of *LuxI* as well as transcription of the *LuxCDABE* gene (Supplementary Figure S6B) (Fuqua and Greenberg, 2002). Transcription and subsequent translation of the *LuxCDABE* gene results in the production of a luciferase enzyme that emits bioluminescence (Supplementary Figure S6C).

Appendix F: Optimization

Reactive Oxygen Species Assay

The ROS assay was optimized over the course of four weeks by slightly modifying the procedure and improving technique. Treating leaf discs is intrinsically difficult. The slightest disturbance of leaf discs by either dispensing the treatment too quickly or nudging the leaves with a micropipette is enough to elicit a wounding response that mimics a MAMP response. Therefore, it is best to avoid elicitation of a wounding response as it detracts from the ability to analyze accrued data. Additionally, leaf discs were arrayed in a 96-well plate and required treatment administration within two minutes of the initial treatment to insure that MAMP responses for all samples were being concomitantly recorded. Thus, each leaf disc was required to be treated within 1.25 seconds and must not be disturbed to prevent elicitation of a wound response.

As a result of these strict parameters, the first two trials failed (Supplementary Figure S7 and S8). As seen in supplementary figure S7, leaf circles treated with water (mock) start with relatively higher light unit (RLU) readings than other treatments. This indicates that a strong wounding response was elicited during treatment. Additionally, the MAMP flg22 failed to elicit the bell-shaped curve characteristic of MAMP responses (Aslam et al., 2009). This indicates that something beyond a simple wounding response caused this trial to fail. It was later discovered that our flg22 had degraded over time, prompting the later use of elf26 as a replacement. .

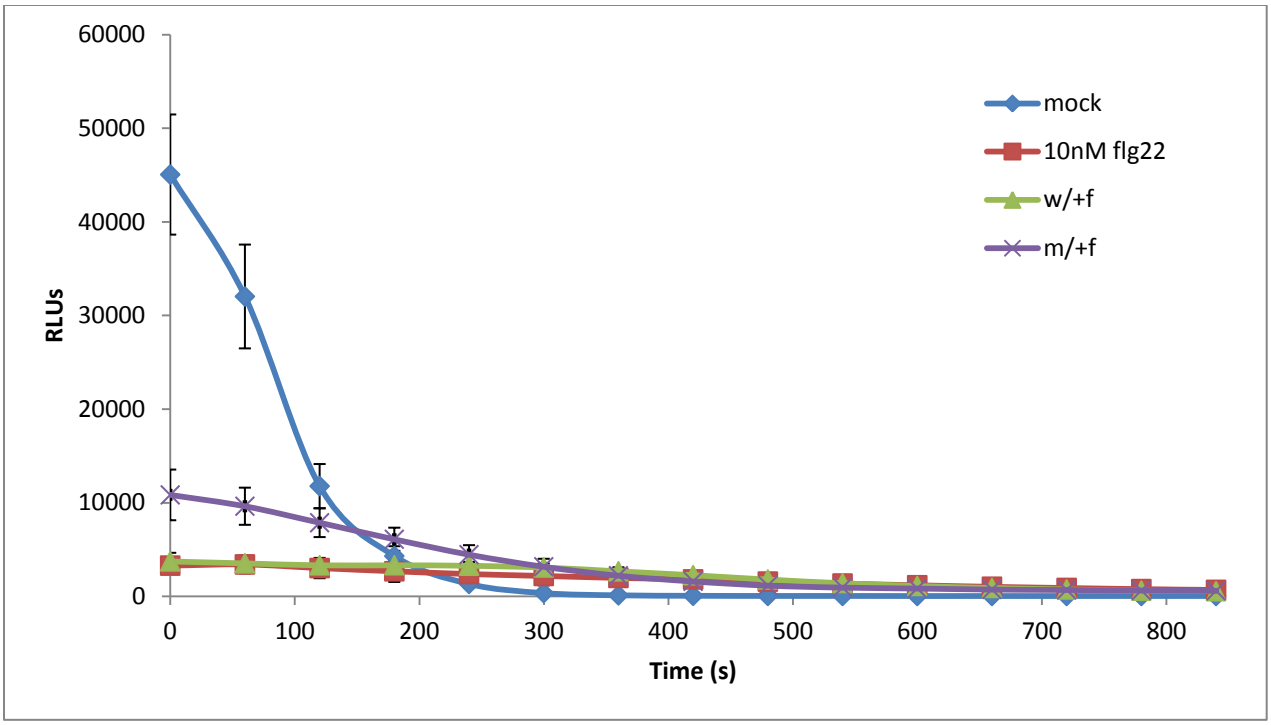


Figure S7 Chemiluminescence emitted by luminol as a byproduct of ROS formation during MAMP responses of *A. thaliana* treated with mock treatment, 10nM flg22, or *P. syringae* pretreated with w/+f or m/+f. *A. thaliana* leaf discs with mock treatment have suffered an obvious wounding response, while *A. thaliana* leaf discs treated with flg22 have suffered a less noticeable wounding response. Error bars are standard error.

Supplementary Figure S8A indicates that a strong MAMP response to elf26 was elicited and wounding response were avoided. However, the MAMP response elicited by elf26 is too strong to determine whether other treatments elicited a response (Supplementary Figure S8A). When the elf26 MAMP response is removed from the graph (Supplementary Figure S8B), it is demonstrated that *A. thaliana* treated with *P. syringae* pretreated with w/+f elicited a weak MAMP response. However, all other treatments fail to elicit any response. The standard error is also so great that no firm conclusions can be discerned from these data.

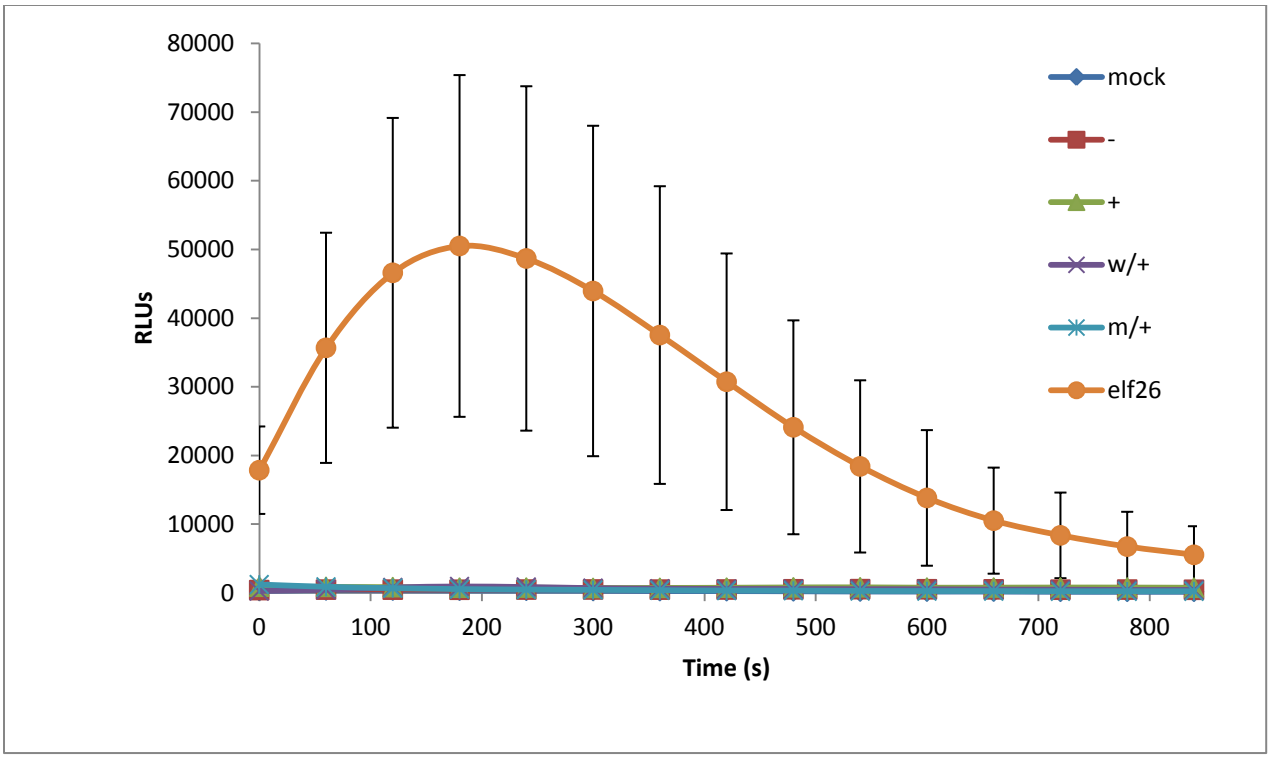


Figure S8A Chemiluminescence emitted by luminol as a byproduct of ROS formation during MAMP responses of *A. thaliana* treated with mock treatment, 100nM elf26, or *P. syringae* pretreated with –f, +f, w/+f, or m/+f. Elf26 elicited the strongest MAMP response from *A. thaliana* leaf discs. Error bars are standard error

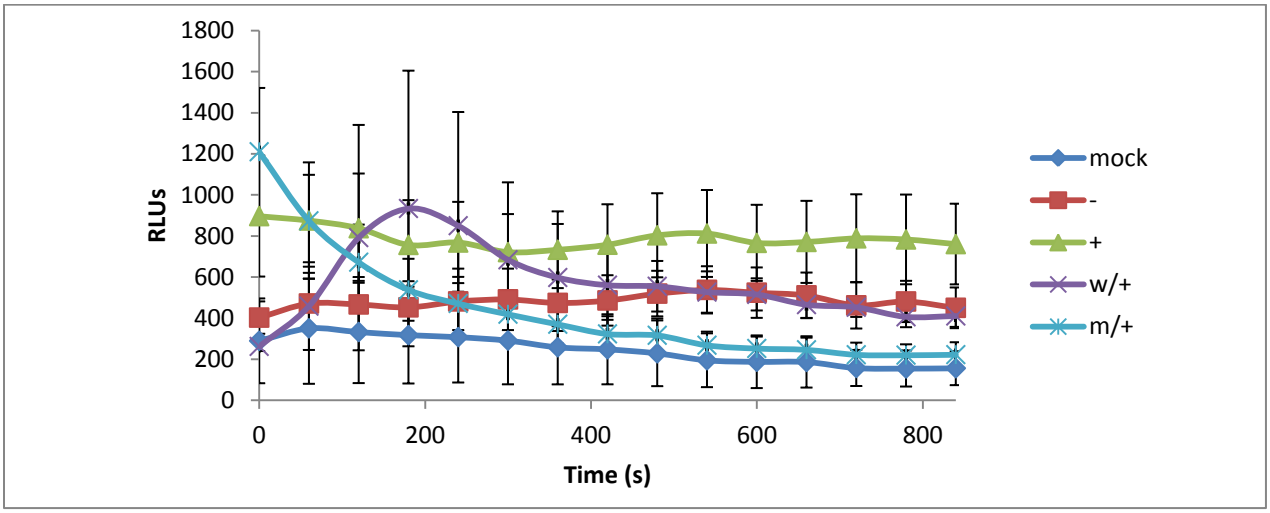


Figure S8B Chemiluminescence of FigureS8A without elf26**.** *A. thaliana* leaf discs treated with *P. syringae* pretreated with w/+ elicited a statistically insignificant MAMP response with respect to other treatments treatments. Error bars are standard error.

After considerable increases in speed and pipetting technique, sources of error as a result of time constraints and disturbances were reduced. However, throughout these experiments residual wounding responses can be seen as initial RLU readings generated at time zero. As time elapses, these

wounding responses diminish. Elicited wounding responses remain a prevalent source of error throughout these experiments because it is nearly impossible to avoid disturbing all leaves.

Luminol was used to report ROS production and horseradish peroxidase was used to strengthen the ROS signal to allow quantification via luminometer. To determine the optimum concentrations of HRP and luminol, various concentrations of luminol, HRP, and bacteria were tested. Ultimately, the concentrations that portrayed the most successful MAMP responses were included in the ROS assay procedure under material and methods. Graphs of failed concentrations are not included as they all have similar shortcomings. Graphs demonstrated either excessive RLU readings or nonexistent readings. Table 1 gives a brief summary of various concentrations and their results.

Trial Number	Bacteria/mL	Luminol Conc. (µM)	HRP Conc. (µL)	Result
1	$1x10^7$	1	1	F
2	$1x10^7$	1	2	F
3	$1x10^7$	2	1	F
4	$5x10^7$	1	1	S-
5	$5x10^7$	1	2	F
6	$5x10^7$	2	1	S-
7	$1x10^8$	1	2	F
8	$1x10^8$	2	1	F
9	$1x10^8$	1	1	S-
10	$1x10^8$	0.5	1	S-
11	$1x10^8$	0.6	1	S-
12	$1x10^8$	0.7 (7mg/1000mL)	1	S

Table S1 Concentrations of bacteria, luminol, and HRP used to determine optimum concentrations. Bacterial concentration was determined using a spectrometer to measure OD_{600}. A failed result is denoted by F, indicating that either chemiluminescence was excessive or nonexistent. S- was used to indicate that chemiluminescence was observed, but there was no distinct MAMP pattern. S indicates a successful result that demonstrates discernible MAMP response-like characteristics.

RLUs were naturally higher in leaves that had been treated. To determine that these RLU readings were naturally higher due to the treatment containing bacteria and not as a result of a wounding response or addition of horse radish peroxidase (HRP) or luminol, bacteria were left absent

from the treatments and leaves were treated with the same chemical compounds without bacteria. Figure S9 demonstrates that even though HRP and luminol are present, there is no difference between the RLU of each treatment with respect to the mock treatment. This indicates that the immediate addition of *P. syringae* intrinsically increases RLU readings. This suggests that the ROS assay is either directly detecting bacterial luminescence or the combination of chemicals with leaf circles and bacteria is generating luminescence.

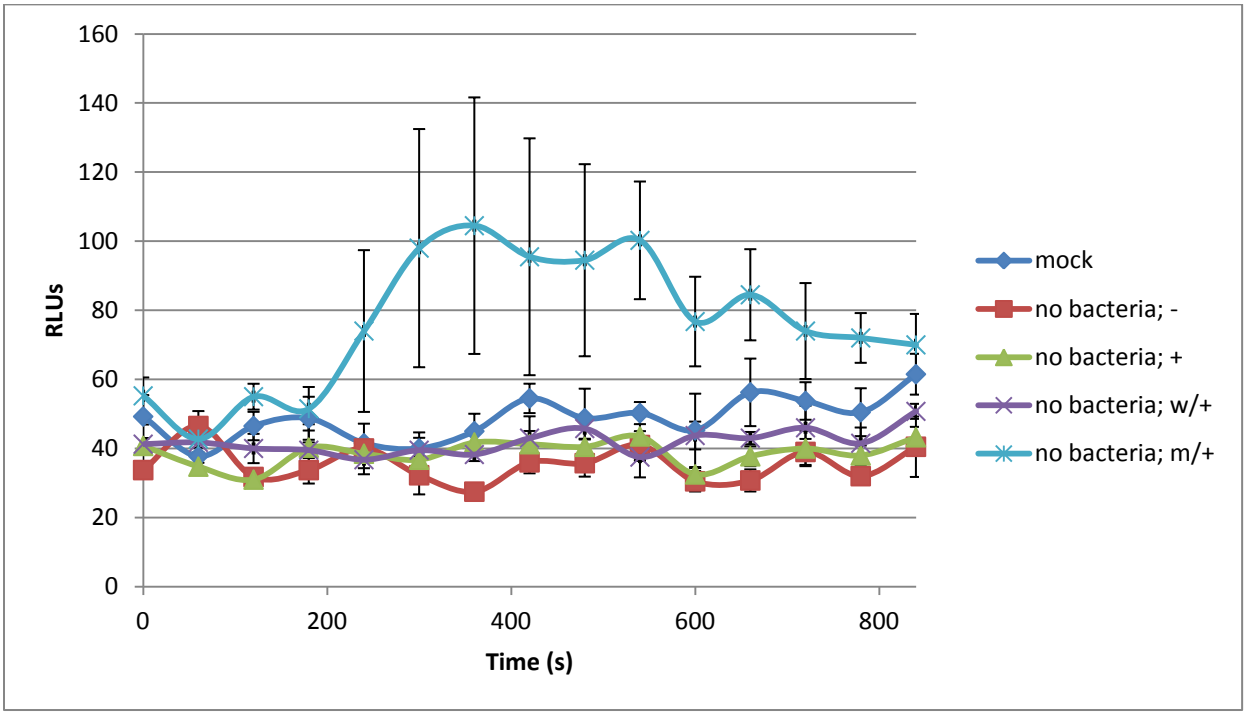


Figure S9 Chemiluminescence emitted by luminol as a byproduct of ROS formation during MAMP responses of *A. thaliana* treated with mock treatment, elf26, or –f, +f, w/+f, m/+f. Error bars are standard error.

After reducing sources of error, finding the proper concentrations, and gaining better understanding as to which components contributed to a peculiarly high initial chemiluminescence, optimization was completed.

Aequorin-Calcium Assay

The aequorin-calcium assay was longer and more involved, but required less time to optimize than the ROS assay. The main difficulty experienced while optimizing this assay was reconstitution of the aequorin with coelenterazine. For various reasons, reconstitution did not always occur (Figure 5). Thus, direct measures of bioluminescence were not adequate for comparing the bioluminescence between individual plants in treatment sample. To account for differential bioluminescence the total bioluminescence by was used to normalize the amount MAMP-induced bioluminescence between individual *A. thaliana* (Figure 5B). With the exception of adding this normalization step, no other optimizations were required.

Appendix G: Supplementary Results

	Chemiluminescence											
	mock		-f		+f		w/+f		m/+f		elf26	
Time (s)	Response	Std Error	Response	Std Error	Response	Std Error	Response	Std Error	Response	Std Error	Response	Std Error
0	2456.39557	1002.819	82.31019	33.60299	103.6102	42.29867	9.777525	3.991658	923.6999	85.80758	1108.511	452.5479
60	2687.42661	1097.137	81.37915	33.2229	109.6383	44.75967	12.62405	5.153747	1083.195	96.06997	1403.138	572.8287
120	2224.22793	908.0372	72.94861	29.78115	102.8463	41.98684	15.92064	6.499573	954.7738	70.58966	1623.212	662.6737
180	1495.85125	610.6787	68.37738	27.91495	85.53303	34.91872	26.0109	10.6189	791.7349	65.03076	1208.852	493.5118
240	809.201005	330.3549	105.4982	43.06945	125.4518	51.2155	92.92147	37.93503	667.074	74.95158	910.11	371.5509
300	553.882539	226.1216	159.6752	65.18712	195.9647	80.00226	149.1837	60.90398	616.0383	99.54798	804.3523	328.3754
360	354.348416	144.6621	193.5593	79.02025	218.597	89.24187	187.8695	76.69742	589.0533	94.97652	711.5584	290.4925
420	274.651537	112.126	191.2019	78.05785	250.7722	102.3773	239.1206	97.62058	533.2733	100.6542	739.4156	301.8652
480	225.417539	92.02632	155.4693	63.47007	229.6566	93.75693	227.7665	92.9853	468.7338	104.1334	760.274	310.3806
540	183.501226	74.91406	128.7753	52.57228	202.5297	82.68239	229.0043	93.49061	403.0689	86.99115	676.7407	276.2782
600	161.815224	66.06079	142.1403	58.02854	186.382	76.09015	170.0478	69.42174	336.8499	87.1711	566.7795	231.3867
660	145.961639	59.58859	132.2024	53.97139	148.8418	60.76439	143.781	58.69833	286.6104	72.97698	415.4924	169.624
720	139.590831	56.98772	136.4357	55.69964	110.8815	45.26717	98.41545	40.17794	205.4231	49.53423	314.1641	128.2569
780	141.532682	57.78048	107.7375	43.98365	87.67535	35.79331	54.97272	22.44252	159.2864	41.77128	227.7399	92.97443
840	137.848709	56.2765	111.353	45.45969	79.38451	32.40859	46.29039	18.89797	122.8283	25.54721	146.1019	59.64585

Table S1 2 Exact measures of chemiluminescence (in relative light units) emitted during the MAMP response of *A. thaliana* treated with mock treatment, elf26, or pelleted *P. syringae* pretreated with –f, +f, w/+f, m/+f. std error refers to standard error, which was omitted in the results section. Values correspond to those plotted in Figure 21. Std error corresponds to error bars in Figure 21. Untreated *A. thaliana* were omitted as standard error was of little consequence with respect to other treatments.

	Chemiluminescence											
	mock		-f		+f		w/+f		m/+f		100nM elf26	
Time (s)	Response	Std Error	Response	Std Error	Response	Std Error	Response	Std Error	Response	Std Error	Response	Std Error
0	1041.167	332.9356	999.1667	44.67903	509.1667	21.84707	2706.833	128.3309	3352.5	264.7543	105.5	9.453174
60	874.6667	317.7061	1143.333	51.70023	592.1667	25.89777	3047.833	127.8966	3711	300.9115	193.5	34.04501
120	612.6667	198.7978	1254.667	58.91134	662	28.36077	3358.333	154.3336	4057.667	338.5025	921.5	164.5455
180	487.3333	127.2865	1358.5	61.02218	693.6667	36.07369	3704.5	188.1661	4464.833	371.6711	1918	262.6863
240	393.1667	88.63762	1389.167	62.70425	768.5	38.76833	3992	185.3342	4782.833	382.4415	2977.833	408.4938
300	298.5	55.21941	1488.5	74.31193	807.3333	46.88971	4269.333	203.7812	5006.667	409.1189	3682.5	489.1623
360	249.6667	43.69109	1546.333	64.98903	869.3333	52.31996	4536.5	207.8934	5143.667	447.2907	4075	581.5681
420	208.5	38.50173	1628.167	88.85023	902.3333	48.12807	4788.5	199.966	5504	456.7316	4232.5	665.0957
480	158.1667	26.6651	1651.5	65.34937	944.6667	42.78759	4967.833	245.2912	5657.833	470.329	4125.167	718.5954
540	136.1667	25.42626	1711.833	78.11625	988.1667	48.03292	5172.5	227.7047	5880.333	474.3781	3992.333	739.0286
600	118.3333	18.72372	1755.167	67.34527	997.8333	53.16604	5313.333	231.0732	5954.167	499.6899	3540.167	682.7127
660	95.83333	13.02156	1839.333	79.91281	1031.167	65.7842	5422	249.0849	6183.667	503.6659	3070.167	603.0534
720	94.16667	10.00472	1872.667	69.49152	1038.5	50.76662	5610.833	275.3655	6257.667	515.6572	2484.667	498.1625
780	78	7.979139	1908.333	83.25853	1058.833	57.63588	5714	257.3609	6404.833	479.8127	1895.833	378.3994
840	76.66667	11.05039	1924.667	90.38079	1118.667	62.97442	5855.667	255.1237	6628.333	508.8415	1352.667	275.8182

Table S2 Exact measures of chemiluminescence (in relative light units) emitted during the MAMP response of *A. thaliana* treated with mock treatment, elf26, or supernatant of *P. syringae* pretreated with –f, +f, w/+f, m/+f. Std error refers to standard error, which was omitted in the results section. Values correspond to those plotted in Figure 22. Std error corresponds to error bars in Figure 21. Untreated *A. thaliana* were omitted as standard error was of little consequence with respect to other treatment.