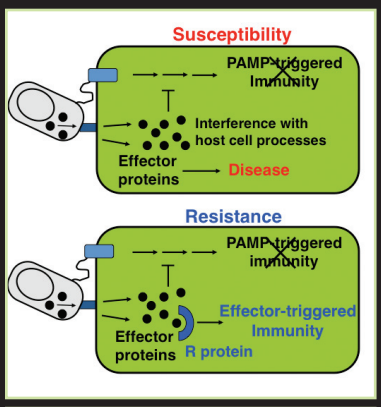
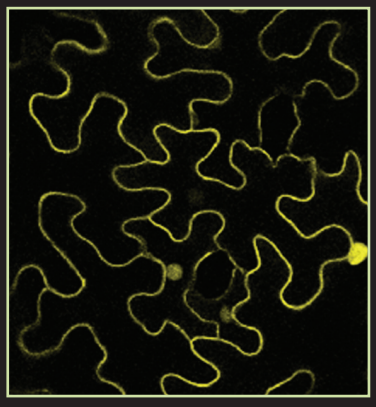
Molecular Plant Immunity





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Preface

Plants and pathogens are constantly engaged in an "arms race," each party competing to develop molecular weapons for the defeat of its enemy. As a result, plants are equipped with a sophisticated immune system for the recognition of invading pathogens, transmission of alarm signals, and rapid activation of efficient defense responses that limit infection. Concurrently, pathogens have developed strategies to cause disease through sabotaging the plant immune system. In an era of growing food demand for the sustainment of the world's population, understanding the molecular mechanisms of plant immunity and microbial pathogenicity is of cardinal importance for devising strategies that limit the large yield losses owing to plant diseases.

This book provides comprehensive coverage of the molecular basis of plant disease resistance by reviewing fundamental features of the plant immune system as well as the most recent insights into this important field of plant biology. Chapter 1 describes recognition of a novel bacterial quorum sensing factor by the rice Xa21 receptor, representing a paradigm for how a first line of immune responses is activated on recognition of conserved molecular signatures of microbial pathogens by plant transmembrane receptors. Chapters 2 and 3 review molecular mechanisms involving resistance (R) proteins, an additional class of immune receptors responsible for the activation of a second line of immune responses. Topics covered in these chapters include structure, control, and activation of R proteins; molecular mechanisms mediating effector recognition by R proteins; and signaling pathways acting downstream of R proteins and leading to the activation of effective immune responses. Chapter 4 describes the role of the plant hormones salicylic acid and jasmonic acid in signaling pathways downstream of immune receptors. Chapters 5, 6, and 7 discuss molecular features of pathogen effector proteins of bacteria, fungi, and oomycetes that interfere with plant immunity and contribute to bacterial and fungal pathogenicity. Chapter 8 presents molecular mechanisms that modulate the interaction between plants and viruses. Chapters 9, 10, and 11 focus on plant-pathogen interactions representing model systems for the interplay between host plants and bacterial, fungal, or viral pathogens. Chapter 12 describes future prospects for genetically engineering disease-resistant plants.

I would like to thank all the authors for their excellent contributions that integrate well-established and emerging concepts to provide an up-to-date review of the state of the art in the challenging field of molecular plant immunity.

Guido Sessa

Molecular Plant Immunity

1 The Rice Xa21 Immune Receptor Recognizes a Novel Bacterial Quorum Sensing Factor

Chang Jin Park and Pamela C. Ronald

Introduction

During the course of evolution, plants and animals have acquired the capability to perceive microbes and respond with robust defense responses. Plant diseases were mentioned in 750 BCE in the Hebrew Bible and again in the writings of Democritus, around 470 BCE (Agrios 1997). Theophrastus made plants and plant disease a subject of systematic studies in 300 BCE. He and his contemporaries believed that plant diseases were a manifestation of the wrath of God (Agrios 1997). Very little useful knowledge about plant diseases was gained for another 2000 years.

The devastating late blight of potatoes, an epidemic that began in 1845 and destroyed the principal food source for millions of people in Ireland, launched the first serious investigations into the basis of plant disease. Although some scientists believed that the causal agent was a microbe (Kelman and Peterson 2002), this hypothesis flew in the face of the prevailing scientific view that microbes commonly found in diseased plant tissues were the products rather than the cause of disease. In 1853, through studies of rusts and smut fungi infection of cereal crops, De Bary conclusively demonstrated that microbes are the causal agents of infectious disease (Agrios 1997).

A quarter century later, the causal role of microorganisms in animal diseases was demonstrated by Koch (1876), who studied anthrax in cattle, using the mouse as a model host. Koch's postulates, developed in the course of these studies, applied equally thereafter to work with plant and animal pathogens. Biffen (1894–1949), a British geneticist and plant breeder, speculated that resistance to disease would be inherited in Mendelian ratios, and in 1905 he demonstrated that this was true for resistance to yellow rust, a fungal disease of wheat (http://www.answers.com/topic/rowland-biffen).

In 1946, Flor (1942, 1971) working with the rust disease of flax proposed the gene-for-gene hypothesis based on genetic analyses of the variation within host and pathogen populations. He used the terms "host resistance genes" and "pathogen avirulence (*avr*) genes." The presence of corresponding *avr-R* genes in each organism leads to recognition and the activation of defense responses, limiting infection. Flor's hypothesis suggested that specific sensors for microbial molecules were present in their hosts. Although some resistance genes conferred broad-spectrum resistance, others did not, specifying resistance to only some races of a particular pathogen species.

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Plants and Animal Immune Systems

Since the discoveries of Biffen >100 years ago, plant breeders have introduced resistance genes into virtually all crops that we eat today. However, for many years, the molecular basis of this resistance remained elusive.

In the 1990s, an avalanche of genetic experiments in numerous laboratories led to the isolation of the first resistance genes from multiple plant species. These discoveries established that diverse molecules and mechanisms govern the resistance phenotypes described in 1946 by Flor. Two of these resistance genes encode cytoplasmic NLRs (nucleotide-binding site domain [NBS], leucine-rich repeat [LRR]–containing intracellular proteins). These include *Arabidopsis RPS2* (*resistance to Pseudomonas syringae 2*) (Kunkel *et al.* 1993; Yu *et al.* 1993), the tobacco mosaic virus resistance gene *N* (Whitham *et al.* 1994), and the flax *L6* gene. These NLR proteins later were shown to perceive directly or indirectly highly conserved effector proteins that target the host immune system.

Other resistance genes isolated at this time encoded the tomato Pto kinase (Martin *et al.* 1993), the rice XA21 receptor kinase (Song *et al.* 1995), and the tomato receptor–like protein Cf9 that lacked a kinase domain (Jones *et al.* 1994). In contrast to the narrow-spectrum resistance conferred by *RPS2, N, L6,* Pto, and *Cf9, XA21* conferred broad-spectrum resistance to the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae (Xoo)* and was predicted to recognize a conserved microbial signature (Ronald *et al.* 1992). The XA21 kinase belongs to a subclass of kinases that carry the non–arginine-aspartate (non-RD) motif (Dardick and Ronald, 2006).

Shortly after the discovery of the first plant resistance genes, work in *Drosophila* established that Toll, originally known for its function in development and its ability to elicit an nuclear factor κB (NF-κB) response, is a key transducer of responses to fungal and gram-positive bacterial infection (Ronald and Beutler 2010). Similar to XA21, Toll carried LRRs in the predicted extracellular domain and signaled through a non-RD kinase called Pelle (which associates with Toll through an adapter protein). Toll also shared the Toll/IL-1 Receptor (TIR) domain with the tobacco N and flax L6 proteins.

Toll does not serve as a receptor for any known molecule of fungal origin. Instead, Toll responds to Spaetzle, which is cleaved from an endogenous protein as a result of infection. This recognition leads to activation of Pelle and to signals that culminate in the production of antimicrobial peptides and hundreds of other proteins, most of unknown function.

In 1998, mouse TLR4 was isolated by positional cloning. TLR4 shared the same structure as Toll. Similar to XA21, TLR4 was predicted to recognize a conserved microbial signature, lipopolysaccharide (LPS), an important component of bacterial cell walls present in most gram-negative bacteria (Poltorak *et al.* 1998). Widely known for its ability to induce septic shock, LPS is a powerful elicitor of inflammation in mammals. TLR4 binds LPS in conjunction with MD-2, a secreted host protein with a hydrophobic pocket into which most of the LPS lipid chains become inserted (Kim *et al.* 2007). An essential contribution to LPS sensing is also made by CD14, an LRR protein that facilitates engagement of LPS by the TLR4/MD-2 complex and is absolutely required for the detection of highly glycosylated (“smooth”) LPS. Similar to Xa21 and Toll, TLR4 also signals through a non-RD kinase. The discovery of a role for Toll and TLR4 in the innate immune response provided a structural link between sensors used by plants and animals to detect infection.

A Plethora of Immune Receptors Recognize Conserved Microbial Signatures

Knowledge about the molecular structures of microbial molecules that elicit an immune response and their cognate receptors provided a critical framework for understanding plant response to

infection (Nürnberger *et al.* 2004; Jones and Dangl 2006; Boller and Felix 2009). This review focuses on receptors of conserved microbial signatures (also called pattern recognition receptors) (Nürnberger *et al.* 2004). These conserved microbial signatures, which are equivalent to animal pathogen-associated molecular patterns (PAMPs), also called microbe-associated molecular patterns (MAMPs), are defined as being (*a*) fundamental to the pathogens' fitness, (*b*) widely distributed within a class of microbes, and (*c*) absent from the host (Medzhitov and Janeway 1997).

Since the discovery of Xa21 (Song *et al.* 1995) and TLR4 (Poltorak *et al.* 1998), a plethora of additional receptors of conserved microbial signatures have been isolated from plants and animals. In plants, in addition to rice XA21 (Song *et al.* 1995), two receptors of conserved microbial signatures have been well characterized: *Arabidopsis* flagellin sensitive 2 (FLS2) (Gomez-Gomez and Boller 2000) and *Arabidopsis* elongation factor (EF)-Tu receptor (EFR) (Zipfel *et al.* 2006). XA21, FLS2, and EFR recognize a sulfated peptide (AxY^S22) derived from the N-terminal region of Ax21 (Lee *et al.* 2009), flg22 peptide derived from bacterial flagellin (Gomez-Gomez and Boller 2000; Zipfel *et al.* 2004), and peptide elf18 derived from the EF-Tu (elongation factor thermo-unstable) protein (Zipfel *et al.* 2006). Many other candidate receptors of conserved microbial signatures have also been isolated or predicted in plant genomes; genomes of monocotyledonous species carry approximately 10-fold more than genomes of the dicots (Schwessinger and Ronald 2012).

In animals, 12 mouse TLRs and 10 human TLRs are now recognized, and most respond to infection, each detecting a specific set of molecules of microbial origin. In flies, only Toll (one of nine paralogs) seems to have a role in the immune response. Mutations that abolish the function of individual TLRs cause selective susceptibility to a certain spectrum of microbes; mutations that prevent all TLR signaling cause severe and general immunodeficiency (Beutler 2009). In addition to the TLRs, animals use RLRs (retinoic acid-inducible gene 1–like receptors) to detect infection by RNA viruses (Satoh *et al.* 2010). Microbial carbohydrates and viral nucleic acids are detected by C-type lectins (dectin-1 and DC-SIGN) and kinases of eIF2α (eukaryotic translation initiation factor 2α) (PKR and GCN2).

Inflammasomes also detect and respond to some pathogens and danger signals (including asbestos, silica, and nigericin) often in a subsidiary, TLR-dependent manner. The cores of these inflammasomes are formed by intracellular proteins of the NOD-like receptor (NLR) family, including Nlrp1, Nlrp3, IPAF, and AIM2. NLR proteins mediate apoptotic and inflammatory responses. The NLR proteins are structurally similar to plant NLR proteins but do not carry TIR domains, which are apparently reserved for signaling by TLRs or IL-1, IL-18, or IL-33, either at the cell surface or within endosomes. In contrast to the animal NLR proteins, none of the plant NLRs has been demonstrated to bind conserved microbial signatures, and they do not associate with non-RD kinases, suggesting a distinct mode of activation for the plant NLR proteins (Dardick and Ronald 2006).

Ax21 Conserved Molecular Signature

Because rice *Xa21* conferred resistance to virtually all *Xoo* strains tested, we hypothesized that it recognized a conserved microbial signature (Ronald *et al.* 1992). We showed more recently that XA21 binds a sulfated peptide, called AxY^S22, derived from the Ax21 (activator of XA21-mediated immunity) protein from *Xoo*. XA21/AxY^S22 binding triggers XA21-mediated innate immunity (Song *et al*. 1995; Lee *et al*. 2009).

The conservation of Ax21 in all sequenced *Xanthomonas spp., Xylella fastidiosa,* and the human pathogen *Stenotrophomonas maltophilia* suggests that Ax21 serves a key biological function. To elucidate this function, we previously isolated and characterized eight *rax* genes (genes required

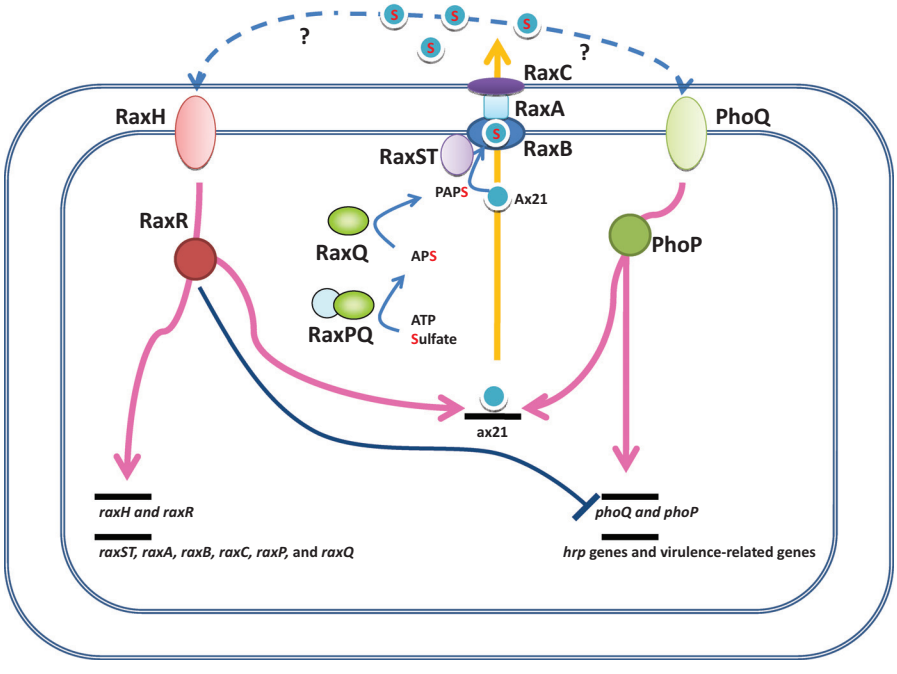

Figure 1.1 Model for Ax21-mediated quorum sensing. Ax21 is sulfated by RaxST, a tyrosine sulfotransferase. As a substrate, RaxST uses 3′-phosphoadenosine 5′-phosphosulfate (PAPS), the production of which is catalyzed by RaxP, an adenosine-5′-triphosphate (ATP) sulfurylase, and RaxQ, adenosine-5′-phosphosulfate (APS) kinase. Sulfated Ax21 is recognized by the rice XA21 receptor. The PhoP/Q two-component regulatory system can sense low concentrations of Mg^{2+} and Ca^{2+} ions present in the environment, which triggers expression of *hrp* and virulence-related genes. This model suggests that the two two-component regulatory systems control different stages of bacterial growth and infection as described in the text. S indicates addition of a sulfuryl group.

for Ax21 activity). *raxA, raxB,* and *raxC* encode components of a predicted type I secretion system (TOSS) (Figure 1.1).

The five other *rax genes include raxST, raxP,* and *raxQ*, encoding enzymes involved in sulfation, and *raxH* and *raxR*, which encode a predicted histidine kinase and cognate response regulator (Shen *et al.* 2002; Burdman *et al.* 2004; da Silva *et al.* 2004; Han *et al.* 2011b). The expression of the eight *rax* genes is density-dependent (Lee *et al*. 2006). Their expression at low densities can be rescued by the addition of high-performance liquid chromatography–fractionated *Xoo* PXO99 supernatants. Fractions from *Xoo* strains lacking Ax21 activity cannot induce density-dependent expression. Therefore, we hypothesized that Ax21 is required for quorum sensing (QS) (Figure 1.1).

QS is a process where small molecules serve as signals to recognize cell population size, leading to changes in expression of specific genes when the QS factor has accumulated to a certain threshold concentration (Fuqua and Winans 1994; Fuqua *et al.* 1994; Waters and Bassler 2005). In gram-positive bacteria, QS is controlled by oligopeptides, whereas gram-negative bacteria generally use acylated homoserine lactones (AHLs) or diffusible signal factors (DSF) for QS (Jayaraman and Wood 2008; Ng and Bassler 2009). One instance of peptide-mediated QS in gram-negative bacteria was reported (Kolodkin-Gal *et al.* 2007).

To determine if Ax21 can serve as a QS factor to regulate density-dependent expression of *rax* genes, we monitored *rax* gene expression in PXO99 and in a mutant strain lacking Ax21

(PXO99Δ*ax21*). We found that the six *rax* genes were highly expressed in PXO99 cultures grown to high population densities (10^8 colony-forming units [CFU]/ml) but not in PXO99Δ*ax21* cultures (Han *et al.* 2011a). Exogenous addition of purified recombinant rAx21 complemented *rax* gene expression in PXO99Δ*ax21,* whereas control fractions did not (Han *et al.* 2011a). These results demonstrate that the mature rAx21 protein is required for QS.

As an additional test to investigate the nature of Ax21, we carried out liquid chromatography–tandem mass spectrometry of supernatants from PXO99Δ*ax21* (rAx21). Nine peptides spanning nearly the entire Ax21 protein except for the predicted N-terminal signal sequence were identified. These results demonstrate that the entire mature Ax21 protein is secreted and that the predicted N-terminal signal sequence is cleaved before secretion.

Bacteria use QS communication to regulate diverse biological processes, including motility, virulence, and transition from a planktonic (free swimming) state to a sessile state, called a biofilm. To elucidate the biological function of Ax21, we compared expression profiles of PXO99 and PXO99Δ*ax21* at three different population densities and found that 489 genes (approximately 10% of the *Xoo* genome) are significantly differentially regulated by Ax21 (Han *et al.* 2011a).

Ten of these genes encode proteins containing the amino acid domains GGDEF, EAL, and HD-GYP. Such proteins have previously been shown to control cyclic diguanylate (c-di-GMP) turnover, a nucleotide-based secondary messenger that regulates diverse microbial phenotypes including growth, motility, virulence, and biofilm formation. In *Xanthomonas spp.,* the RpfC/G sensor kinase and response regulator are required for DSF perception and signal transduction leading to c-di-GMP degradation through a protein containing an HD-GYP domain (Dow *et al*. 2006). In the opportunistic pathogen *Pseudomonas aeruginosa,* AHL-mediated c-di-GMP production is regulated by a tyrosine phosphatase (TpbA) (Ueda and Wood 2009). Thus, three distinct QS systems (AHL-mediated, DSF-mediated, and Ax21-mediated) control the expression of genes encoding proteins that regulate c-di-GMP turnover. Bacterial c-di-GMP has also more recently been shown to trigger the innate immune response of mouse and human cells (Karaolis *et al.* 2007; McWhirter *et al.* 2009).

Our expression analysis also identified a set of genes that are upregulated by Ax21 during early log phase (Han *et al.* 2011a). These include the *gumE, gumJ,* and *gumK* genes, which encode proteins required for biosynthesis of xanthan gum, an important component of the *Xanthomonas* extracellular polymeric substance (EPS) (Crossman and Dow 2004). EPS enables bacteria to adhere to each other or to a solid surface, a key component of biofilms.

To assess if Ax21 is required for biofilm formation, we examined biofilm formation in the PXO99, PXO99Δ*ax21,* and PXO99Δ*raxST* strains using a plate adherence assay. The PXO99Δ*ax21* strain formed significantly fewer biofilms compared with the PXO99 strain. Exogenous addition of purified rAx21 restored biofilm formation in PXO99Δ*ax21*. Aggregation assays comparing PXO99Δ *ax21* and PXO99 revealed that Ax21 is also required for *in vivo* aggregation of *Xoo* (Han *et al*. 2011a). These experiments demonstrate that Ax21-mediated QS controls biofilm formation in *Xoo*.

Our microarray data also revealed that at early log phase, Ax21 upregulates expression of genes involved in bacterial motility. To test whether Ax21 controls *Xoo* motility, we assayed the phenotype of *Xoo* PXO99 and PXO99Δ*ax21* strains using a swimming motility plate assay. We found that the motility of PXO99 was twofold higher than the motility of PXO99Δ*ax21* (Han *et al.* 2011a) indicating that Ax21 regulates *Xoo* swimming motility on semisolid media.

We previously showed that the predicted histidine kinases PhoQ and RaxH are required for Ax21-mediated activities (Burdman *et al.* 2004; Lee *et al.* 2008b). We hypothesized that one of these proteins was the bacterial receptor for Ax21. In support of this hypothesis, we observed that biofilm formation in both the PXO99Δ*raxH* and PXO99Δ*phoQ* strains is reduced compared with the PXO99 strain. We next tested whether biofilm activity could be rescued by addition of purified

rAx21 protein to these mutant strains. We found that PXO99Δ*phoQ* but not PXO99Δ*raxH* could form biofilms after complementation with rAx21 (Han *et al.* 2011a).

The observation that Ax21 is required for QS and for control of density-dependent expression of genes involved in motility, c-di-GMP turnover, and biofilm formation suggests that PXO99Δ*ax21* strains would be impaired in virulence. However, earlier experiments indicated no significant changes in virulence phenotypes when PXO99Δ*ax21* infection was tested by clipping rice leaves with bacteria dipped in high-density cultures (10^8 CFU/ml) (Kauffman *et al*. 1973; Lee *et al*. 2009). Because under field conditions, *Xoo* infection through hydathodes or wounded sites requires only a low inoculation density (10^4 CFU/ml) to initiate infection (Mizukami 1961), we hypothesized that an effect of Ax21 on virulence has been masked by the high-density inoculation approach.

To test this hypothesis, we established a new inoculation method. *Xoo* strains PXO99, PXO99Δ*raxST,* and PXO99Δ*ax21* strains were cultured in PSA (peptone sucrose media) plates and diluted with water to 10^3 CFU/ml. Unclipped rice leaves were soaked in bacterial suspensions for 2 days, and bacterial populations were assessed 2 days after inoculation. We found that the population of the wild-type PXO99 strain was twofold higher than that of the PXO99Δ*raxST* and PXO99Δax21 strains using the low-density soaking method (Han *et al.* 2011a). In contrast, the populations of all three strains were similar 2 days after inoculation using the high-density scissor clipping method. These results indicate that *ax21* and *raxST* are required for full virulence during early stages of infection that mimic field conditions.

To investigate the mechanism with which Ax21 regulates motility, virulence, and biofilm formation, we generated *Xoo* strains mutated for 12 genes that are regulated by Ax21. Virulence of five strains was partially or completely lost in the knockout mutants. Six strains displayed a reduction in biofilm formation, and 11 strains partially lost swimming motility. These analyses indicate that Ax21 exerts its complex control through the regulation of target genes.

The discovery that a small protein from a gram-negative bacterium has a dual role in QS and in activation of the host innate immune response has not previously been demonstrated. However, we do not believe this is an anomaly or that the biological importance of Ax21 is restricted to plant pathogens. For example, we previously reported that Ax21 is also conserved in the nosocomial pathogen *Stenotrophomonas maltophilia* and proposed a similar role for Ax21 in this species (Lee *et al.* 2009). Consistent with our hypothesis, a synthetic Ax21 protein was shown to regulate gene expression, motility, and biofilm formation in *S. maltophilia,* extending our findings to an animal pathogen (McCarthy *et al.* 2011).

These results suggest that not only do these other gram-negative bacteria use N-terminal processed small proteins for QS, but also that some of the hundreds of predicted receptors in rice and other species, for which no corresponding conserved microbial signature has yet been identified, detect such molecules (Dardick and Ronald 2006). Such knowledge can be used to develop reagents to immunize hosts against infection or antagonists to disrupt QS-mediated virulence activities and biofilm formation (Swem *et al*. 2008), a process thought to be involved in 65%–80% of bacterial infections of plants and animals (Davies 2003).

Post-translation Modification of Ax21 Influences Biological Activity and Recognition by XA21

Tyrosine sulfation is one of the most abundant post-translational modifications (Kehoe and Bertozzi 2000). In contrast to phosphorylation, which regulates processes that occur inside the cell, sulfated proteins/peptides are typically directed to the outside of the cell where they modulate cell-cell and ligand-receptor interactions.

A notable example pertinent to agriculture is sulfation of the *Sinorhizobium meliloti* Nod factor that is required for specific recognition by its host alfalfa (Roche *et al.* 1991). In humans, sulfation of residues in the C-terminus of the α subunit of the hCG (human chorionic gonadotropin) ligand is required for binding with the N-terminal LRR domain of the hCG receptor (Bielinska 1987; Bhowmick *et al.* 1996). Another example of regulation of receptor-ligand reactions controlled by sulfation is the binding of the gp120 subunit of the envelope glycoprotein of human immunodeficiency virus (HIV) to the human chemokine co-receptors CD4 and CCR5. Sulfation of tyrosine residues in the N-terminal segment of CCR5 appears to be critical for both HIV-1 entry and binding of gp120-CD4 complexes (Farzan *et al.* 1999, 2000).

We have shown that a 17-amino acid synthetic peptide containing a sulfated tyrosine-22 (AxYS22), derived from Ax21, binds the XA21 receptor and is sufficient for triggering XA21-mediated immunity. In contrast, peptides lacking tyrosine sulfation are inactive (Lee *et al.* 2009). Thus, AxYS22 represents a previously uncharacterized type of conserved microbial signature recognized by host receptors: a sulfated peptide. Isolated field strains that lack the predicted secretion or sulfation components are only weakly virulent in plants carrying *XA21* (Choi *et al.* 2003; da Silva *et al.* 2004). This suggests that the sulfated Ax21 protein is required for bacterial fitness under field infection conditions and that *Xoo* evades XA21-mediated recognition by altering secretion or post-translational modification of the Ax21 protein, or both.

Ax21 tyrosine sulfation on Tyr22 is mediated by the sulfotransferase encoded by *raxST* (Han and Ronald, unpublished data) (Figure 1.1). Similar to *Pseudomonas* ORF1, which is critical for glycosylation and determines host specificity, *raxST* can be considered the avirulence determinant because strains lacking *raxST* are not recognized by XA21 and are virulent in rice greenhouse tests. The specificity conferred by a post-translational modification, supports an emerging theme for conserved microbial signatures – that sequence variation and post-translational modifications such as glycosylation, acetylation, and sulfation can modulate receptor recognition (Taguchi *et al.* 2003; Kunze *et al.* 2004; Sun *et al.* 2006). In the XA21/Ax21 system, the AxY^S22 peptide sequence is invariant in all sequenced *Xanthomonas* species. Sulfation provides specificity to the system, just as flagellin recognition in plant species is modulated by glycosylation (Takeuchi *et al.* 2003).

PhoPQ Two-Component Regulatory System Detects and Responds to Extracellular Nutrient Status and Controls Expression of **hrp** *Genes*

Pathogens have evolved integrated regulatory circuits that control the coordinated expression of one set of genes in one environment and a different set of genes in another environment. In pathogenic bacteria, these regulatory circuits are generally controlled by two-component systems (TCSs), composed of histidine kinases (HKs) and response regulators (RRs). In response to environmental stimuli, HKs phosphorylate cognate RRs, which then activate gene expression (Charles *et al.* 1992).

In *Salmonella typhimurium,* PhoQ activity is modulated by extracellular levels of Mg^{2+} and Ca^{2+}. Low cation concentrations promote activation of *mgtA, pbgC, pcgF, pcgG, mgtCB,* and *psiD* genes, whereas high concentrations result in repression of these genes (Vescovi *et al.* 1996; Cheung *et al.* 2008). These results indicate that *Salmonella* PhoQ is a sensor for periplasmic concentrations of divalent cations. The role of divalent cations as signals for PhoQ is also supported by the crystal structures of the PhoQ periplasmic sensor domains from *S. typhimurium* and *E. coli* (Cheung *et al.* 2008). Similarly, we have shown that the *Xoo* PhoPQ system is required for sensing low extracellular Mg^{2+} and Ca^{2+} concentrations, conditions that the pathogen likely is confronted with on entry into the xylem of the rice plant (Lee *et al.* 2008b). In addition, we showed that Ax21 activity is impaired

in a *phoQ* knockout strain (PXO99Δ*phoQ*) as reflected by enhanced growth of this strain in rice lines carrying XA21 (i.e., increased susceptibility) (Lee *et al.* 2008b). These data suggest that PhoQ not only senses divalent cations but also regulates Ax21 activity.

Which biological activities are controlled by the PhoP/Q regulatory system? We have reported that a *phoP* knockout strain (PXO99Δ*phoP*) is impaired in *Xoo* virulence and is no longer able to activate the response regulator HrpG (hypersensitive reaction and pathogenicity G) in response to low levels of Ca^{2+} (Lee *et al.* 2008b). The impaired virulence of the PXO99Δ*phoP* strain can be partially complemented by constitutive expression of *hrpG,* indicating that PhoP/Q controls a key aspect of *Xoo* virulence through regulation of *hrpG*. These results are reminiscent of the fact that the PhoP/Q TCS is required for virulence in *Shigella flexneri* and *Yersinia pestis* (Moss *et al.* 2000; Oyston *et al.* 2000).

In *Xanthomonas* spp. and *R. solanacearum,* HrpG activates *hrpX* and *hrpA* expression. HrpX upregulates the expression of the *hrpB* to *hrpF* operons, which encode components of a type III secretion system (T3SS). T3SS secretes proteins directly into host cells. HrpX also controls expression of type III effectors (T3E), which are proteins secreted via T3SS (Merighi *et al.* 2003; Wei *et al.* 2007). We showed that expression of *hrpA* and *hrpX* in *Xoo* is significantly higher in the presence of low Ca^{2+} concentrations in the wild-type strain but not in the PXO99Δ*phoP* strain (Lee *et al.* 2008b). These results demonstrate that the PhoP/Q TCS senses cation concentrations to regulate *hrp* gene expression through HrpG.

Because we have shown that RaxR negatively regulates *phoP* gene expression (Burdman *et al.* 2004), we hypothesized that *hrpG, hrpA,* and *hrpX,* which are positively regulated by PhoP, would be negatively regulated by RaxR. We found that 23 *hrp* and *hrp*-related genes, including *hrpG, hrpA,* and *hrpX,* are downregulated in RaxR overexpression strains and upregulated in the PXO99Δ*raxR* strain (Lee *et al.* 2008b). These results support a model in which *Xoo hrp* gene expression is under control of PhoP, which is negatively regulated by the RaxR/H TCS (Figure 1.1). According to this model, the *Xoo* PhoP/Q TCS works in partnership with RaxR/H to assess population density and control the regulation of effectors. Our results suggest the presence of an integrated regulatory circuit that the bacterium uses to respond to environmental fluctuations.

These findings led to the hypothesis that Ax21 triggers a transition from a quiescent or epiphytic state to an invasive or pathogenic state of the bacterium in response to changing extracellular conditions sensed by the two TCSs. This hypothesis would explain why the PhoP/Q TCS, which triggers expression of a set of genes, including *hrp* genes, through the negative regulation of RaxR/H, is also required for Ax21 activity. Because *Xoo* must monitor population size under changing conditions, an integrated and flexible response system is desirable. In this model, *Xoo* can sense low concentrations of Mg^{2+} or Ca^{2+} in the host via the PhoP/Q TCS. These conditions would trigger *phoP*-regulated gene expression. The consequence would be an increased expression of genes required for virulence such as *hrp* genes. Bacteria would propagate, resulting in accumulation of Ax21, which is sensed by the RaxR/H TCS. This perception would lead to upregulation of *rax* genes and repression of *hrp* genes.

Non-RD Receptor Kinase Xa21

Non-RD Kinase Domain

XA21 is a receptor kinase that consists of LRR, transmembrane, juxtamembrane (JM), and intracellular kinase domains (Song *et al.* 1995) (Figure 1.2). Kinases are classified as arginine-aspartate

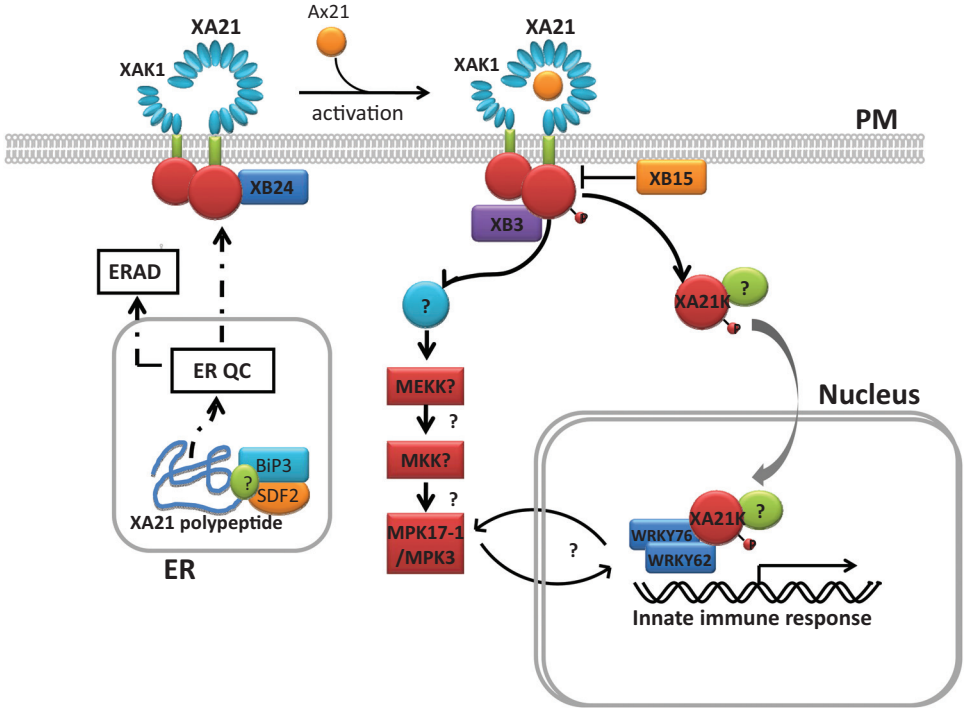

Figure 1.2 Model for XA21-mediated immunity. The ER chaperone and co-chaperone BiP3 and SDF2 are involved in XA21 biogenesis (Park *et al.* 2010). XA21 is processed through the ER and translocated to the PM where it binds the XA21-associated kinase 1(XAK1)(Chen *et al.* Submitted). XB24 physically associates with XA21, keeping the XA21 protein in an inactive state (Chen *et al.* 2010b). Ax21 binding to XA21 activates its cytoplasmic non-RD kinase, inducing dissociation of XA21 from XB24 (Lee *et al.* 2009; Wang *et al.* 1998; Chen *et al.* 2010b). Autophosphorylated Thr705 transfers its phosphoryl group to other XA21 residues, activating XA21 (Chen *et al.* 2010a). Activated XA21 is cleaved and translocates to the nucleus (Park and Ronald, 2012). XA21 is hypothesized to transphosphorylate downstream target proteins that have not yet been identified. XB3 may serve to activate a downstream MAPK cascade (Wang *et al.* 2006). In the nucleus, XA21 binds WRKY transcription factors that regulate defense-related genes, such as *PR1* and *PR10*, either positively or negatively (Peng *et al.* 2008, 2010). Recruitment of XB15 to Ser697 in the XA21 JM domain and subsequent dephosphorylation of phosphorylated residues attenuates the XA21-mediated immune response.

(RD) or non-RD kinases. RD kinases carry a conserved arginine (R) immediately preceding the catalytic aspartate (D) (Dardick and Ronald 2006). In contrast to RD kinases, non-RD kinases typically carry a cysteine or glycine in place of the arginine. It was previously reported that non-RD kinases are associated with the control of early signaling events in both plant and animal innate immunity (Dardick and Ronald 2006). For example, in humans, recognition of PAMPs at the cell surface is largely carried out by TLRs (Nürnberger and Brunner 2002). TLR1, TLR3, TLR5, TLR6, TLR7, TLR8, and TLR9 associate with the non-RD interleukin-1 receptor associated kinase (IRAK) family (Akira and Takeda 2004), and TLR3 and TLR4 associate with the non-RD receptor interacting-protein (RIP) kinases (Meylan *et al.* 2004) via adapter proteins.

In plants, receptors of conserved microbial signatures carry kinases of the non-RD class (Dardick and Ronald 2006). Plant genome analyses have revealed the presence of a large family of the non-RD receptor kinases at the cell surface, with >30 encoded in the *Arabidopsis* genome and >320 found

in the rice genome (Dardick and Ronald 2006). In addition to the rice XA21 receptor, members include the *Arabidopsis* PRRs FLS2 and EFR (Gomez-Gomez and Boller 2000; Zipfel *et al.* 2006), the barley PRG1 (resistance to *Puccinia graminis* f. sp. *tritici*) (Brueggeman *et al.* 2002), and the rice XA26, Pid2 (Sun *et al.* 2004; Chen *et al.* 2006). The predicted intracellular receptor of conserved microbial signature, wheat WKS-1, also carries a non-RD kinase (Fu *et al.* 2009).

The activation mechanism of RD kinases, which carry a conserved arginine immediately preceding the catalytic aspartate in subdomain VIb (Johnson *et al.* 1996; Nolen *et al.* 2004), is well studied. Most require phosphorylation of the activation segment for full kinase activity (Nolen *et al.* 2004). The phospho group in the activation segment coordinates the positively charged amino group of the arginine, leading to stabilization of the otherwise highly flexible activation segment, and enhances enzymatic activity.

In non-RD kinases, an uncharged amino acid, usually a cysteine or glycine, replaces the arginine of RD kinases, suggesting a different mechanism of activation (Dardick and Ronald 2006). Several different regulatory mechanisms have been observed for mammalian non-RD kinases, such as relief of autoinhibition by C-terminal extension (Kobe *et al.* 1996; Shin *et al.* 2011) or tyrosine phosphorylation in the P+1 loop immediately downstream of the activation segment (Mayans *et al.* 1998). The crystal structures of several non-RD kinases reveal a highly ordered conformation of the activation segment in the absence of phosphorylation (Nolen *et al.* 2001; Tereshko *et al.* 2001; Scheeff *et al.* 2009; Shin *et al.* 2011). These results suggest that non-RD kinases are constitutively active, which might represent a general theme of non-RD kinase regulation. Support for this hypothesis comes from more recent results showing that the ATPase XB24 promotes XA21 autophosphorylation, holding it in a biologically inactive state. Only on ligand binding does the ATPase disassociate, triggering XA21 activation (Chen *et al.* 2010a, b) (Figure 1.2).

Arabidopsis FLS2 and EFR and rice XA21 display relatively weak kinase activity *in vitro* compared with their co-regulatory RD kinase counterparts and with other RD kinases involved in development (X. Chen *et al.* unpublished data). In addition, the kinase activity of the non-RD kinases IRAK1, RIP-1, RIP-2, RIP-4, and XA21 is at least partially dispensable for their function in immunity (Wang *et al.* 1998; Andaya and Ronald 2003; Ronald and Beutler 2010). In contrast, the catalytic activity of several co-regulatory RD kinases, such as IRAK4 (Janssens and Beyaert 2003), RIP-3 (Cho *et al.* 2009; He *et al.* 2009), and AtBAK1 (Roux *et al.* 2011; Schwessinger *et al.* 2011), appears to be critical for their function. Taken together, these observations suggest that at least part of the function of non-RD kinases is to serve as phosphorylation-dependent scaffold proteins (Dardick and Ronald 2006; Good *et al.* 2011).

XA21 LRR Domain

An *Xa21* family member called *Xa21D* is able to confer partial resistance to *Xoo* and confers the same broad-spectrum resistance, suggesting that recognition of Ax21 also triggers XA21D-mediated resistance. *Xa21D* encodes a predicted secreted extracellular soluble protein with an LRR domain 98% identical to that of XA21 (Wang *et al.* 1998). In contrast to XA21, XA21D lacks both TM and intracellular signaling relay domains. How does an exclusively extracellular-localized receptor induce an intracellular signaling cascade? XA21D potentially works analogously to MD2, an extracellular soluble protein necessary for LPS perception in mammals. LPS binding to MD2 induces a heterocomplex formation with subsequent TLR4 intracellular signaling (Akashi-Takamura and Miyake 2008).

In animals, microbial-induced receptor dimerization is often required for transphosphorylation and the activation of downstream signaling pathway. Heterodimerization or homodimerization