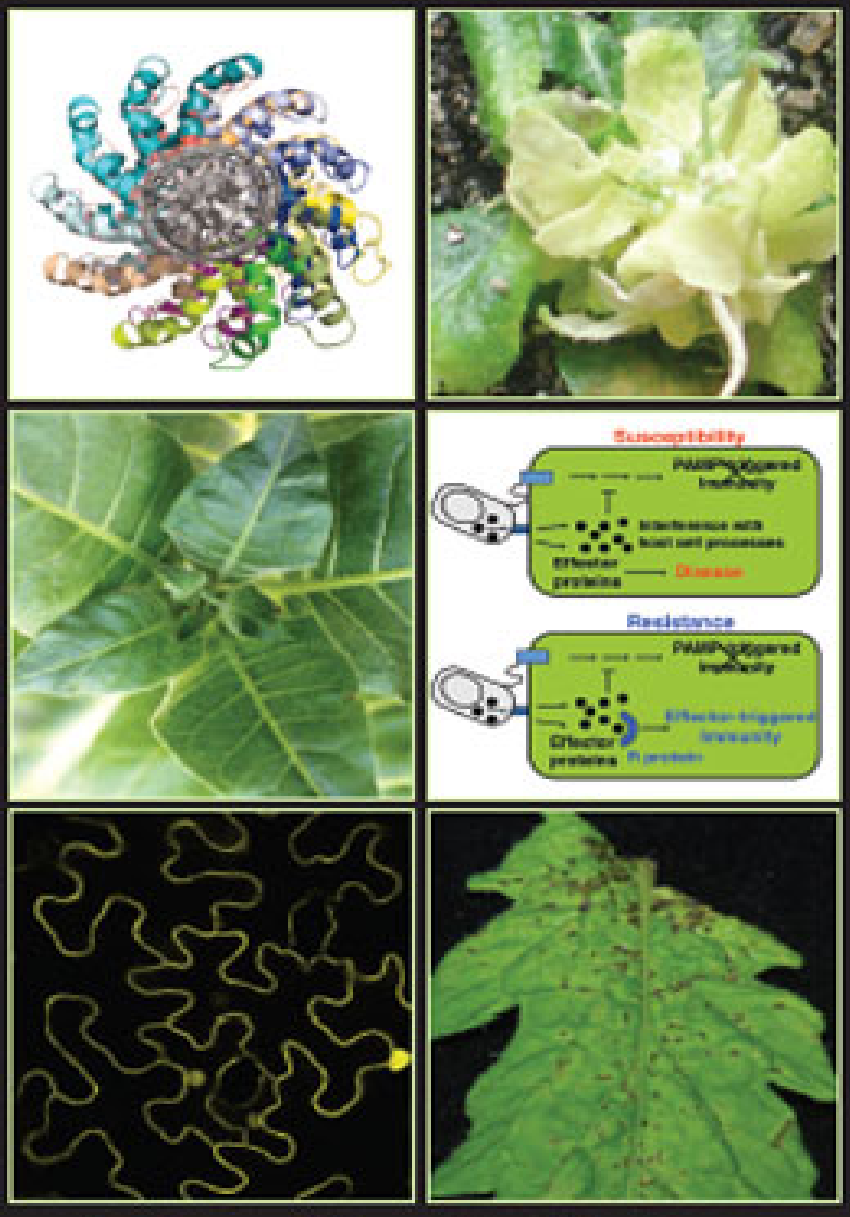
Molecular Plant Immunity



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Contents

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Editor
GUIDO SESSA
Department of Molecular Biology and Ecology of Plants
Tel-Aviv University
Tel-Aviv, Israel

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Contributors

Douglas Baker
Department of Plant Pathology
University of California at Davis
Davis, California, USA

Adam J. Bogdanove
Department of Plant Pathology and Microbiology
Iowa State University
Ames, Iowa, USA

John P. Carr
Department of Plant Sciences
University of Cambridge
Cambridge, United Kingdom

Yan-Jun Chen
Department of Plant Biology and Biotechnology
University of Copenhagen
Copenhagen, Denmark

Gitta Coaker
Department of Plant Pathology
University of California at Davis
Davis, California, USA

David B. Collinge
Department of Plant Biology and Biotechnology
University of Copenhagen
Copenhagen, Denmark

Ben J. C. Cornelissen
University of Amsterdam
Faculty of Science, SILS
Molecular Plant Pathology
Amsterdam, The Netherlands

Pierre J. G. M. de Wit
Laboratory of Phytopathology
Wageningen University
The Netherlands

Feng Feng
State Key Laboratory of Plant Genomics
and National Center for Plant Gene Research
Institute of Genetics and Developmental Biology
Chinese Academy of Sciences
Beijing, China

Amy Wahba Foreman
Department of Biological Sciences
University of South Carolina
Columbia, South Carolina, USA

Aaron W. Hummel
Department of Plant Pathology and Microbiology
Iowa State University
Ames, Iowa, USA

Aardra Kachroo
Department of Plant Pathology
University of Kentucky

Lexington, Kentucky, USA

Pradeep Kachroo Department of Plant Pathology
University of Kentucky
Lexington, Kentucky, USA

Michael F. Lyngkjær Department of Plant Biology and Biotechnology
University of Copenhagen
Copenhagen, Denmark

Lisong Ma University of Amsterdam
Faculty of Science, SILS
Molecular Plant Pathology
Amsterdam, The Netherlands

Gregory B. Martin Boyce Thompson Institute for Plant Research
Ithaca, New York, USA

Bilal Ökmen Laboratory of Phytopathology
Wageningen University
The Netherlands

Chang Jin Park Department of Plant Pathology and the Genome Center
University of California at Davis
Davis, California, USA

Gail J. Pruss Department of Biological Sciences
University of South Carolina
Columbia, South Carolina, USA

Pamela C. Ronald Department of Plant Pathology and the Genome Center
University of California at Davis
Davis, California, USA

Thierry Rouxel INRA-Bioger
Thiverval-Grignon, France

Frank L. W. Takken University of Amsterdam
Faculty of Science, SILS
Molecular Plant Pathology
Amsterdam, The Netherlands

Brett M. Tyler Center for Genome Research and Biocomputing
Department of Botany and Plant Pathology
Oregon State University
Corvallis, Oregon, USA

Vicki Vance Department of Biological Sciences
University of South Carolina
Columbia, South Carolina, USA

Harrold A. van den Burg University of Amsterdam
Faculty of Science, SILS

Molecular Plant Pathology
Amsterdam, The Netherlands

André C. Velásquez Department of Plant Pathology and Plant-Microbe Biology
Cornell University
Ithaca, New York, USA

Jack H. Westwood Department of Plant Sciences
University of Cambridge
Cambridge, United Kingdom

Jian-Min Zhou State Key Laboratory of Plant Genomics
and National Center for Plant Gene Research
Institute of Genetics and Developmental Biology
Chinese Academy of Sciences
Beijing, China

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

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.

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 ($AxY^{s}22$) 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).

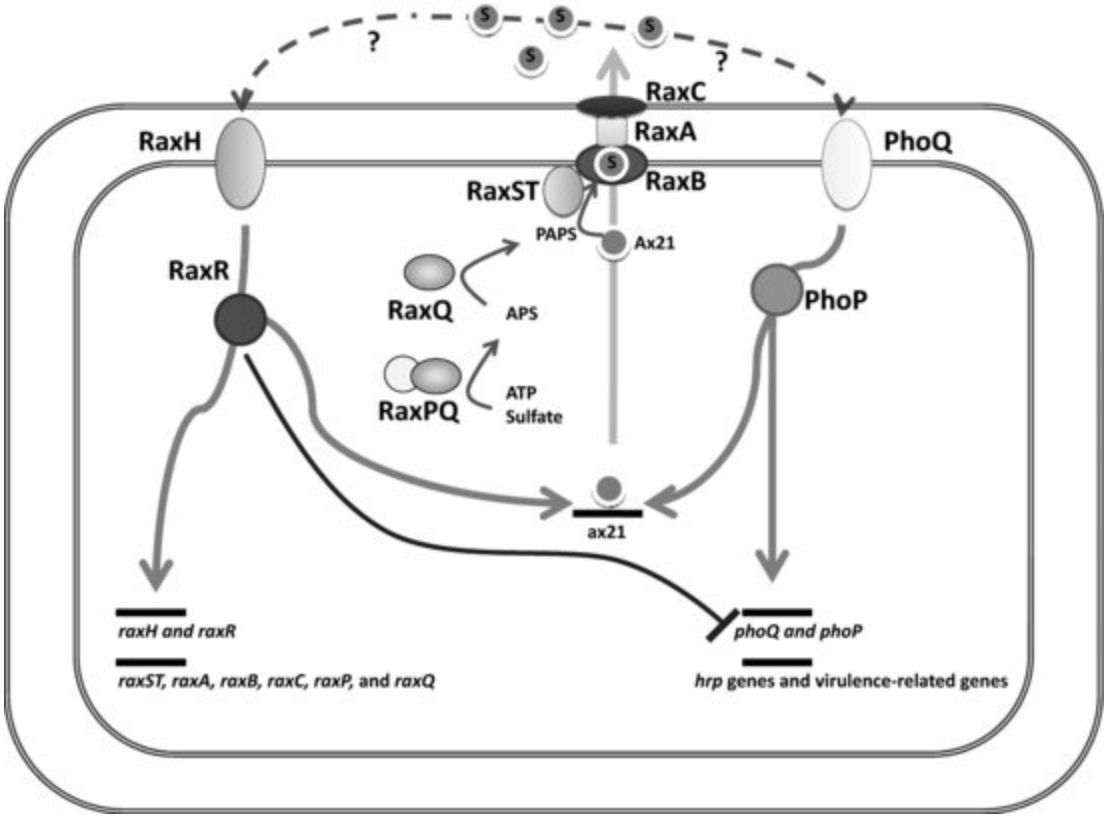
Ax21 Conserved Molecular Signature

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^{s}22, derived from the Ax21 (activator of XA21-mediated immunity) protein from *Xoo*. XA21/AxY^{s}22 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 for Ax21 activity). *raxA, raxB,* and *raxC* encode components of a predicted type I secretion system (TOSS) (Figure 1.1).

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.



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 (PXO99*Δax21*). 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 PXO99*Δax21* 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 PXO99*Δax21* 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 PXO99*ΔraxH* and PXO99*ΔphoQ* 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 PXO99*ΔphoQ* but not PXO99*ΔraxH* 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 PXO99*Δax21* strains would be impaired in virulence. However, earlier experiments indicated no significant changes in virulence phenotypes when PXO99*Δax21* 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