GENE EXPRESSION AND SIGNALING IN *Rxo1* GOVERNED INNATE IMMUNITY IN CEREALS

by

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Abstract

Many maize lines carry *Rxo1*, an *NB-LRR* gene that confers a rapid hypersensitive response (HR) after infiltration with the rice streak pathogen *Xanthomonas oryzae* pv. *oryzicola* (*Xoc)* or the maize stripe pathogen *Burkholderia andropogonis (Ba)* carrying the effector genes *avrRxo1* or *avrRba1* respectively. Interestingly, when expressed as a transgene in rice, *Rxo1* also confers a strong and rapid HR to *Xoc* strains harboring the *avrRxo1* type III effector gene.

To gain insights into the *Rxo1* signaling network, we used a combination of functional genomics and bioinformatics, molecular genetics and reverse genetics.

Microarray experiments were carried out to investigate the temporal expression profiles of nonhost and host responses to isogenic strains of *Xanthomonas oryzae pv. oryzae* (*Xoo*), the rice bacterial blight pathogen, and *Ba* with and without the *Xoc* type III secreted effector gene *avrRxo1*.

Xoc AvrRxo1 induces disease resistance in maize when delivered by *Xoo* or *Ba*. We show that recognition of the two bacterial pathogens is translated into similar transcriptional outputs. Cluster analyses revealed that *Xoo* and *Ba* co-regulated genes display different kinetics and amplitudes and showed that gene clusters are associated with overrepresentation of known and putative novel DNA cis regulatory elements. One early induced gene, *ZmPti1b,* is a serine threonine kinase. RNAi-mediated gene silencing of a rice ortholog of *ZmPti1b, OsPti1a,* revealed that *OsPti1a* is required for *Rxo1*-governed resistance.

Using a full length coding sequence as bait to screen a yeast-two-hybrid library, we identified 11 rice proteins that interact with RXO1. Functional analysis of two showed that *Os1PVOZ*, encoding a putative transcription factor, is required for *Rxo1*-dependent HR whereas *OsATL6*, a putative RING finger type E3 ubiquitin ligase gene is dispensable. Scanning of the rice genome for putative DNA binding sites suggests that Os1PVOZ is a master regulator of many signal transduction pathways, including those that mediate plant defense responses.

Our investigations identified key signaling components that mediate *Rxo1*-specified resistance and possibly resistance mediated by other *R* genes.

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Dedication

This thesis is dedicated to The Ever Shinning Light of My Soul

Serigne Djily Abdou Fatha Falilou Mbacke.

CHAPTER 1-Literature review

Introduction

Plants perceive, interpret, and respond to cues from different environments that exist on our planet. They represent an important source of food for humans, animals, insects, and micro-organisms. Rice is one of the most important food resources in the world, and this is particularly true for many developing countries. In Asia, many high yielding hybrids are susceptible to *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*), the causal agent of bacterial leaf streak , and no source of simply inherited resistance is available is available in rice (1). Many maize lines however carry *Rxo1*, an *NB-LRR* resistance gene that confers a rapid hypersensitive response (HR) upon challenge with *Xoc* or with the maize and sorghum bacterial stripe pathogen *Burkholderia andropogonis* (*Ba*). These bacterial pathogens are specifically recognized by plants that carry the *Rxo1* gene because the pathogens carry avirulence effector genes *avrRxo1* or *avrRba1*, respectively (2). Moreover, the rice bacterial blight pathogen, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), when transformed with the *Xoc avrRxo1*, elicits the HR on *Rxo1* maize but not on maize without *Rxo1*. Interestingly, when expressed as a transgene in rice, the maize *Rxo1* gene confers a rapid and strong HR upon challenge with *Xoc* or *Xoo* carrying the *Xoc avrRxo1* type III effector gene (3). Understanding the molecular basis underlying plant immunity will help in devising strategies for crop improvement through alteration of biological pathways including interspecies transfer of resistance genes to rewire signaling networks that mediate resistance.

In this present work, we set out to investigate the regulatory networks mobilized by cereal plants expressing *Rxo1* in response to rice bacterial blight and maize bacterial stripe pathogens carrying the *Xoc* type III effector gene *avrRxo1*. We also set out to identify early signaling components that translate AvrRxo1 recognition into defense execution. To this end, we used a combination of functional genomics and bioinformatics, molecular genetics and reverse genetics. The following review will highlight current progress in understanding interactions between plants and pathogens result in induction of signal cascades that lead to resistance.

Plant pathogens generally interfere with plant development and reproduction. Phytopathogenic bacteria for example, gain access to the intercellular spaces and the vascular system via stomata, hydatodes, and wounds. In these environments, they multiply and differentiate a needle-like structure, the so called type III secretion (TTSS) system that breaches the cell wall and inserts directly into the host cytoplasm. The TTSS delivers an arsenal of effector molecules inside the living plant cell to subvert host metabolism and to promote pathogen fitness (4).

Plants possess highly effective innate immunity that can be divided into two main overlapping branches. The first of these relies on surface localized pattern recognition receptors (PRRs) to sense pathogen associated molecular patterns (PAMPs) also referred to as microbe-associated molecular patterns (MAMPs) (4-7). This first line of inducible defense responses active against diverse microbes is referred to as PAMP triggered immunity (PTI) (4, 7). The second major layer of inducible defense, known as effector triggered immunity (ETI), involves the recognition of pathogen effectors by plant resistance proteins (R proteins) (4, 7) and the elicitation of strong and rapid immune responses often associated with a programmed cell death, the so called hypersensitive response (HR). A detailed understanding of the molecular bases underlying plant innate immunity will help in guiding the translation of fundamental knowledge into crop improvement for food, nutraceutical, and bioenergy.

Recognition of bacterial pathogens and elicitation of PAMP-Triggered-Immunity (PTI)

Upon recognizing an invading pathogen, plants deploy a variety of both local and systemic defense responses that ultimately lead to restriction of pathogen ingress. Plants possess hundreds of receptor-like kinases (RLKs) with divergent extracellular domains that provide ligand specificity and intracellular kinase domains involved in signal transduction (8, 9). Roles for most RLKs are unknown. Many are implicated in growth and development and others function as PRRs (10, 11). Plants sense would-be pathogens through the detection by PRRs of diverse PAMPs that include bacterial flagellin, cold-shock protein (CSP), and elongation factor-Tu (EF-Tu), fungal chitin, and the oomycete elicitor INF1 (12–16). Despite their diversity, different PAMPs activate similar sets of responses, including activation of a MAP kinase (MAPK) cascade, a burst in active oxygen species (AOS), the regulation of a similar set of

genes, and cell wall reinforcement (11, 14, 15, 17,18). Hence, signaling pathways initiated by different PAMPs converge on a common set of outputs that define and conclude PTI.

Signaling in PAMP triggered immunity (PTI)

Research towards understanding the molecular bases that govern PTI is still in its infancy as compared to ETI. However, work in the past few years has shed some light into PAMP recognition and signaling. Most of our knowledge regarding PTI comes from studies with two PAMPs, flagellin and EF-Tu. Most plant species recognize flg22, a highly conserved 22 amino acid epitope within the N-terminus of the flagellin protein (19). Recognition of flg22 is achieved through the single pass transmembrane leucine-rich repeat receptor-like kinase (LRR-RLK) FLAGELLIN-SENSING 2 (FLS2) (20). EF-Tu is perceived by a receptor of the same subfamily as FLS2, the LRR-RLK-EF-Tu receptor (EFR) (11). Global gene expression profiling indicated no evidence for subsets of genes specific to flagellin or EF-Tu regulation (11). A model for PTI signaling is shown in Fig. 1

Upon flg22 binding to the extracellular LRR domain of FLS2, FLS2 heterodimerizes with a second LRR-RLK that positively regulates responses to both flg22 and EF-Tu, the brassinosteroid receptor associated kinase 1, BAK1 (21, 22). *Nicotiana benthamiana BAK1* silenced plants are compromised in responses to diverse PAMPs in addition to flg22, including bacterial CSP and oomycete INF1, suggesting that BAK1 also regulates the function of their corresponding but unknown PRRs (22). Flg22 binding, ultimately leads to receptor endocytosis from the cell surface and this internalization requires both kinase activity and the ubiquitination-related (PEST) motif in the C-terminal domain of the Arabidopsis FLS2, AtFLS2 (23).

Several members of the MAP kinase (MPK) and WRKY transcription factors families are downstream components of PTI signaling (18 and Fig. 2). MPK3 and MPK6 are rapidly activated in response to flg22 in *Arabidopsis* protoplasts and this activation requires a kinase-active FLS2 (18). A third MPK, MPK4 is also activated in response to flagellin (24). Transient overexpression of constitutively active versions of MPK kinase (MP2K) in *Arabidopsis* protoplasts suggested that two MP2Ks, MKK4 and MKK5 but not MKK1 nor MKK2, could activate MPK3 and MPK6 (18). Recent genetic evidence however concluded that MKK1 can indeed activate all three MPKs and is a positive regulator of defense responses to bacterial

elicitor flagellin and to various *Pseudomonas syringae* avirulence effector proteins (28). Furthermore, *Arabidopsis* T-DNA knockout mutants indicated that a MP2K kinase (MP3K), MEKK1 is required for flg22 induced activation of the negative defense regulator, MPK4 but not MPK3 or MPK6 (29). *MEKK1* mutants display a salicylic acid (SA) dependent dwarf phenotype, constitutive expression of defense related genes and callose deposition. All the responses associated with *MEKK1* mutation are interestingly independent of its kinase activity, suggesting this MP3K may play a structural role in PTI signaling (29). A yeast-two-hybrid screen has indeed shown that MEKK1 associates with MKK1 and 2 which in turn interact with MPK4 and that the N-terminal regulatory domain of MEKK1 can also interact with MPK4 but not MPK3 or 6 (30, 31). Furthermore, suppression of MEKK1 kinase activity delayed the hypersensitive response triggered by *Pseudomonas syringae* AvrRpt2 (29). Taken together, these studies show evidence that there is no linear pathway for a given MPK module, but rather an interconnected network of signaling cascades that need holistic genetic and biochemical approaches to gain more insight into the global picture of PTI, this frontline of defense that can halt further pathogen colonization.

Molecular bases of effector-triggered susceptibility (ETS) and functions of type III effectors

Successful pathogens have evolved strategies to subvert host immunity and to bring about disease susceptibility. Gram negative bacteria use the TTSS to translocate a toolkit of effector proteins inside the host cell to enhance pathogen fitness (32-37). There have been significant breakthroughs recently that have increased our understanding of type III effector function (4, 37, 35, 36). And as the scheme unfolds, it is clear that a detailed understanding of the effector functions and the functions of their host protein and gene targets holds significant clues as to how bacterial pathogens subdue host signaling and commandeer host metabolism to their own benefit.

The lack of easily scored phenotypes and the observation that deletion of single effectors seldom affects virulence have hindered investigations into understanding the virulence functions of most type III effectors (37, 38). Therefore, much of this research has focused on specific aspects of plant defense including cell wall reinforcement, the HR, ethylene (ET) and jasmonate (JA) signaling, and flagellin-dependent defense responses.

Several *P. syringae* TTSS effectors have been described that suppress defense responses elicited either by *TTSS*-defective mutant bacteria or flg22. These include AvrPto, AvrRpm1, AvrRpt2, and HopAI1, to mention only a few (4, 39-42 see Fig.2). Some *Xanthomonad* type III effectors, on the other hand, activate expression of the so called host susceptibility genes. They belong to the *transcription activator-like* (*TAL*) effector gene family, also known as *avrBs3* family, that hijack the plant transcriptional machinery and promotes disease susceptibility (43, 44). Many of the features involving type III effector activities have been reviewed by Grant and colleagues (32). The following section will therefore focus on recent work involving some of the above mentioned effectors.

In planta expression of the *P. syringae avrPto* or *avrPtoB* effector genes promote growth of otherwise non pathogenic bacteria and suppress callose deposition induced by *TTSS* deficient bacteria or by flg22 (39, 45). Deletion of *avrPto* and/or *avrPtoB* reduces bacterial virulence in *Arabidopsis* (46). Transient expression of *avrPto* and *avrPtoB,* but not other effectors such as *avRpm1*, *avrRpt2*, or *avrB,* suppresses flg22-dependent expression of early defense marker gene, *FRK1* (*FLG22-INDUCED RECEPTOR LIKE KINASE*), in *Arabidopsis* protoplasts (46). These observations strongly suggest that different effectors use distinct mechanisms to suppress PTI. Consistent with this idea, both AvrPto and AvrPtoB but not AvrRpt2 or HopAI1 completely abolish flg22-induced activation of MPK3 and MPK6 (45). Moreover, these two effectors act upstream of the MP2Ks, MKK4 and MKK5 and the MP3K, MEKK1, as gain of function mutants of these protein kinases abrogate AvrPto and AvrPtoB mediated MPK inhibition (46). AvrPto is a small protein that is targeted to the plasma membrane via a myristoylation process, and mutations that disrupt its membrane localization also disrupt its ability to suppress flg22-induced MPK activation and *FRK* induction (46, 47).

AvrPto interacts with a tomato kinase called Pto, a cytosolic protein involved in ETI, in yeast-two-hybrid assays (48). The crystal structure of an AvrPto-Pto complex was recently solved and biochemical analyses show that AvrPto occupies the P+1 loop and a second loop within Pto, thereby inhibiting its kinase activity in vitro (49). The Pto kinase domain architecture is reminiscent of the intracellular kinase domain of RLKs, but it lacks the extracellular LRR domain (50). Recent studies have shown that AvrPto binds to FLS2 and EFR PRRs and inhibits their kinase activity to block immune responses in plant cells (35). Moreover, transgenic *Arabidopsis* plants expressing the *avrPto* gene display phenotypes that phenocopy

brassinosteroid insensitive mutants characterized by severe growth and reproduction defects. This observation led to the hypothesis and the demonstration that not only AvrPto, but also the sequence unrelated AvrPtoB effector proteins bind to the brassinosteroid receptor associated kinase 1, BAK1, a common signaling component of both PTI and brassinosteroid signal transduction pathways (21, 22, 51). This binding disrupts the formation of a flagellin-induced complex with the flagellin receptor FLS2 (36). Furthermore, *avrPto* and *avrPtoB* mutants that fail to suppress PTI also fail to interact with BAK1. Remarkably, 30 minutes after challenging *Arabidopsis* plants with *P. syringae*, FLS2 forms a complex with BAK1. This complex formation is further enhanced by a *TTSS* deficient and *avrPto*/*avrPtoB* double mutant and disrupted ed by wild type bacteria expressing the two effectors (36). Both *avrPto* and *avrPtoB* are found in several *P. syringae* isolates infecting different plant species (52), suggesting an important role for these virulence factors in the pathogenesis and the adaptation of this pathogen to diverse host plants. AvrPtoB is a modular protein. While its N-terminal domain binds to Pto and several RLKs, its C-terminal domain displays a crystal structure showing striking similarities to U-box and RING-finger type of E3 ubiquitin ligases (53). AvrPtoB exhibits ubiquitin ligase activity towards a second member of the Pto family that is also involved in ETI, the FEN kinase, and targets it for degradation by the proteasome, thereby suppressing ETI in addition to its PTI suppressing function (54). Hence, AvrPtoB suppresses two major layers of plant innate immunity. It is very likely that AvrPtoB might use the plant ubiquitin-proteasome pathway to target more host proteins for degradation in addition to Fen.

Several effectors act at different steps of PAMP signaling cascades (55). The *P. syringae* HopAI1 protein for instance, interferes with flg22 induced immunity by directly dephosphorylating MPK3 and MPK6, two key components of the FLS2 signal transduction pathway. HopAI1 is however not a protein phosphatase, but irreversibly inactivates these MPKs through a unique phosphothreonine lyase activity, thereby eliminating rephosphorylation of the inactivated MPKs and suppressing AOS production, *pathogenesis-related* (*PR*) gene expression, and callose deposition (56). Whether or not this effector targets other MPKs or other phosphorylated proteins remains to be determined. HopAO1 is another effector that acts on phosphorylated proteins through its tyrosin phosphatase activity and suppresses *PR gene* expression and ETI (57, 58). Targets for this effector are unknown.

The ability to interfere with the function of multiple host proteins is likely not restricted to only some effectors. *P. syringae* type III effector proteins AvrB, AvrRpm1, and AvrRpt2 are translocated inside *Arabidopsis* cells during infection. AvrB and AvrRpm1 are acetylated in the host cell upon delivery and targeted to the plasma membrane (59). The presence of both leads to phosphorylation of the host protein RIN4, the RPM1 interacting protein 4 (60). AvrRpt2 is a cysteine protease that undergoes autoprocessing and activation leading to RIN4 cleavage and further elimination by the proteasome (61-64). Hence, three sequence-unrelated type III effectors target the same host protein using at least two different mechanisms to alter its physiological state.

RIN4 is a small plant protein that localizes to the plasma membrane probably through palmitoylation and/or prenylation (60, 64-66). *In planta* overexpression of *RIN4* inhibits PTI responses and its absence enhances these responses, suggesting *RIN4* is a negative regulator of this defense pathway (40). The elimination of RIN4 by AvrRpt2 is inconsistent with RIN4 being a virulence target for this effector as far as PTI is concerned. More *Arabidopsis* proteins bear sequences similar to the proteolytic cleavage sites observed in AvrRpt2 and RIN4 and several of these proteins were cleaved in an AvrRpt2-dependent manner in transient *in planta* expression system (63). Moreover, protease deficient AvrRpt2 significantly reduce the ability of *P. syringae* to grow on *rin4* mutant plants, suggesting the presence of other virulence targets for this effector (63). Genetic analysis of these putative AvrRpt2 target proteins will help in gaining more insight into the mechanism by which this effector enhances *P. syringae* virulence.

In addition to interfering with PTI signaling, bacterial effectors can also target the plant defense transcriptome. Members of the TAL family of effector proteins possess plant nuclear localization signals (NLS), leucine zipper (LZ) and acidic transcriptional activation domains (AD), and modulate expression of host genes (67, 68). In susceptible hosts, the *Xanthomonas campestris* pv. *vesicatoria (Xcv)* AvrBs3 effector protein causes hypertrophy of mesophyll cells and contribute to pathogen dispersal between pepper plants in the field (69, 70). *Xcv* AvrBs3 forms homodimers in plant cells (71) and is targeted to the nucleus (69) where it binds to the upa-box (**Up**regulated by **A**vrBs3) and activates transcription of various host genes including *Upa20*, a putative transcription factor of the *basic helix-loop-helix* (*bHLH*) family, a master regulator of cell size (72).

Three other genes in rice are specifically induced in response to the bacterial blight pathogen, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), carrying *PthXo1*, *PthXo6*, and *PthXo7 TAL* effector genes. *Os8N3*, which is a member of the *MtN3* gene family, is specifically induced in the presence of PthXo1 and is required for disease susceptibility as silencing of this gene renders otherwise susceptible plants, highly resistant to the rice blight pathogen (44). *OsTFX1*, encoding a bZIP transcription factor, and *OsTFIIγ1* a small subunit of the general transcription factor TFIIA, are specifically induced in response to *Xoo* carrying *PthXo6* and *PthXo7,* respectively. *Xoo PXO86* strain does not grow well on rice IRBB5 cultivar, nor does it induce the expression of *OsTFIIγI*. Remarkably, transgenic expression of a plasmid carrying *PthXo7* enables *PXO86* not only to induce *OsTFIIγI* but to also proliferate in rice tissues and cause significant higher lesions (43). On the other hand, a knock out mutation in *PthXo6* impairs *Xoo* virulence while the ectopic overexpression of *OsTFX1* reinstates full virulence of the pathogen (43). Whether or not these three effectors bind directly to cis elements within the promoters of these host genes is unknown. These data suggest however that some TAL effectors activate the expression host genes promoting a favorable environment for the pathogen to thrive and multiply.

Overall, the above described investigations into understanding the functions of type III effectors reveal that bacterial pathogens subdue host immunity and manipulate host growth and development at different levels, including signal perception, transduction, and output.

Recognition of avirulence effector proteins and Effector-Triggered-Immunity (ETI)

Plant pathogenic bacteria including *Pseudomonas* and *Xanthomonas* live in the plant apoplast where they traffic a suite of effector proteins through the type III secretion system inside host cells to promote parasistism. Type III effectors are, however, double edged swords, their biochemical activities betray their presence and allow for R proteins to specifically recognize them and initiate ETI, the second major layer of inducible defenses (32). Their virulence features hence translate into an avirulence (avr) activity in the presence of R proteins. Most *R genes* encode NB-LRR (Nucleotide Binding Site-Leucine-Rich-Repeat) proteins. The signaling events required to activate NB-LRR-mediated resistance are poorly understood. Modeling studies based on the NB-ARC structure of animal NOD-like receptors (NLRs) reminiscent of plant R proteins specifying ETI suggest that NB-LRR proteins shuttle between

ADP bound inactive and ATP bound active states (73, 7). It is possible that R proteins are maintained in an inactive state through intra (74, 75) and inter-molecular interactions whereas type III effectors disrupt the inactive complex, promote ADP exchange for ATP thereby activating downstream signaling. Several NB-LRR proteins are known to associate with host proteins that are targeted by type III effectors (6, 7, 35).

The tomato Pto kinase for example constitutively associates with the R protein Prf locking this NB-LRR protein into an inactive state. Two loops that interact with AvrPto are required in Pto to hold Prf inactive, suggesting that AvrPto triggers ETI by relieving the inhibitory activity of Pto on Prf (76, 49). Recognition of AvrPtoB is also dependent on Prf and requires Pto or the Fen kinase. The N-terminal domain containing amino acids 1-307 is sufficient to trigger Prf/Pto dependent resistance while amino acids 1-387 are needed to elicit Prf/Fen-mediated ETI. Through its ubiquitin ligase activity however, AvrPtoB C-terminal domain promotes Fen degradation to evade ETI (53, 54, 77). These observations suggest a functional mimicry whereby, the host has evolved a ubiquitination resistant form of Fen in the form of Pto to allow AvrPtoB recognition and the reinstatement of immunity. Likewise, Pto may mimic the kinase domain of PAMP RLKs targeted by AvrPto to enable recognition and ETI.

As described earlier, RIN4 is targeted by at least three different *Pseudomonas* proteins. In the absence of effectors, RIN4 forms complexes with the RPM1 and RPS2 NB-LRR R proteins negatively regulating their activity (78, 79). RIN4 cleavage by the AvrRpt2 cysteine protease activates RPS2 dependent-resistance, while AvrB and AvrRpm1 induced-phosphosphorylation of RIN4 elicits RPM1 dependent defense responses (80). RIN4 may also be a decoy in acquiring AvrRpt2 cleavage sites also present in many *Arabidopsis* proteins that may be the true virulence targets for this type III effector.

A remarkable recognition mechanism is achieved by the *Bs3 R* gene which encodes a flavin monoxygenase. This *R* gene accommodates the very cis-element found in the promoter of host susceptibily genes targeted by the *Xanthomonas* AvrBs3 protein, the upa box. Transcriptional activation of *R gene* in this case initiates ETI (81).

The findings that are described above, suggest that some host genes and proteins have evolved to mimic pathogen virulence targets and act as decoys that mediate effector recognition and ETI.

Signaling downstream *R* gene activation

Upon specific recognition of an Avr protein by the cognate R protein, several biochemical, molecular, and physiological alterations ensue as an outcome. These events include an elevation in AOS termed the oxidative burst, the activation of calcium, H^+/K^+ fluxes and PKs, an accumulation of plant hormones such as SA, JA, and ET, the induction of *PR genes*, the biosynthesis of antimicrobial compounds (phytoalexins), and the strengthening of plant cell wall (82, 83). These defense responses often culminate into a type of programmed cell death (PCD), the HR, at the infection site to limit pathogen spread but also the induction of systemic acquired resistance to prime further infection against a broad spectrum of pathogens (84, 85).

The NB-LRR R proteins can be subdivided into two subclasses based on their N-terminal domains. The first of which displays homology to the Toll and interleukin-1 receptor (TIR) and the second exhibits a predicted coiled-coil (CC) domain (86). These two classes show some difference in their requirements for downstream regulators. Several CC-NB-LRR R proteins signal through NDR1 (Non Disease Resistance1), a putative glycosylphosphatidylinositol (GPI)-anchored protein, whereas many TIR-NB-LRR require EDS1 (Enhanced Disease Susceptibility1), a lipase-like protein (87). There is however some exception to this dichotomy, because some CC-NB-LRR proteins seem to signal through an additional pathway (88, 89). Many R protein-dependent signaling pathways also differ in their requirement for key regulators of ubiquitination-dependent protein degradation, RAR1 and SGT1 (90, 91).

Diverse R/Avr signaling pathways often converge and lead to the expression of similar sets of genes including signaling molecules and molecules associated with defense execution and metabolism. SA, JA and ET are key signal molecules that mediate the expression of defense response genes (92). More recently the abscisic acid (ABA) hormone has been implicated in immune responses (93, 94). The role of these hormones in defense has been studied by genetic analysis of mutants affected in their biosynthesis and/or perception or mutants with altered expression of marker genes for these pathways.

Ectopic expression of the bacterial *SA hydroxylase* gene (*NahG*) that hydrolyses SA into catechol in plants compromises resistance (95). More importantly, several mutants were isolated that do not accumulate SA or respond to exogenous SA application. Both types exhibit enhanced susceptibility to pathogens (96-98). Likewise, JA/ET insensitive mutants or mutants that fail to accumulate these two signal molecules also show increased susceptibility (99, 100). Conversely,

mutants that accumulate high levels of SA, display spontaneous lesion formation reminiscent of the hypersensitive response, constitutively express several *PR* genes, and acquire enhanced resistance against pathogens (101-103). Moreover, the SA insensitive mutant, *npr1*, is also JA/ET insensitive, providing a link between SA and JA/ET signal transduction pathways (104). Exogenous application of ABA increases plant susceptibility to pathogens, suggesting ABA is a negative regulator of defense responses. Furthermore, mutant plants compromised in their ability to synthesize or to respond to ABA, express increased resistance against pathogens, and show constitutive expression of the JA/ET defense marker gene *PDF1-2*, suggesting that ABA antagonizes the JA/ET signaling pathway (93, 94). The SA pathway was shown to be preferentially deployed against biotrophic pathogens whereas the JA/ET pathway was induced in response to necrotrophs (105). Signaling interactions between these pathways are summarized in Fig. 3.

Taken together, these observations show that signal molecules do not work alone, they talk to each other, they influence each other in negative, positive, or synergistic ways, and they branch to form networks that culminate to bring about a fine tuned response to cues emanating from the environment.

The maize *Rxo1* gene: A sentinel for both host and nonhost resistances

Interplay between host and nonhost interactions

Resistance exhibited by an entire plant species to all isolates of a microbial species is referred to as non-host or species resistance (reviewed in ref 106). Nonhost resistance is thought to be the most common mechanism by which plants ward off would be pathogens. This type of resistance, considered to be the most durable form of plant disease resistance is governed by an interplay of both constitutive barriers and inducible reactions that can be divided into two types (107). While type I nonhost resistance does not display any visible symptoms, type II nonhost resistance results in rapid HR reminiscent of host resistance often controlled by *R* genes. The latter is usually governed by single *R* genes whose products recognize pathogen *avr* gene products and trigger a rapid and strong HR (108). Nonhost resistance displays strong similarities with host resistance but it remains unclear whether the same mechanisms are involved in these two types of immunity.

Type I nonhost resistance relies on both preformed and inducible defenses. The plant cytoskeleton provides a physical barrier against many pathogens as disruption of actin filaments results in loss of resistance to several nonhost fungi (109). Many secondary metabolites that plants constitutively produce possess antimicrobial activities. Saponin-deficient mutants of a diploid oat species, *Avena strigosa*, are compromised for disease resistance against the nonhost fungal pathogens *G. graminis* var. *tritici* and *Fusarium culmorum* (110). Inducible type I nonhost resistance is believed to be mediated by PAMP recognition (106). The *Arabidopsis NHO1* (*nonhost1*) gene encoding a glycerol kinase is induced by several *P. syringae* isolates from bean and tobacco and is required for resistance against these bacteria and against the fungal pathogen *B. cinerea* (111). Moreover, overexpression of *NHO1* results in enhanced resistance to the otherwise fully virulent isolate *P. syringae* DC3000 on *Arabidopsis*. The nonhost pathogen *P. syringae* pv. *phaseolicola* elicits a wide array of plant defense genes, similar to those induced during host ETI but without the visible symptoms of the HR (112).

Type II nonhost resistance on the other hand culminates into the visible symptoms of the HR. A functional TTSS is required for *P. syringae* pv. *phaseolicola* to cause nonhost HR on tobacco (113), suggesting the recognition of type III effectors by R proteins to bring about type II nonhost resistance. Similarity between host and nonhost resistance mediated by *R* genes is illustrated by the requirement of the *SGT1* gene by both types of immune responses (114).

A central question is: If type I nonhost resistance is sufficient to confer immunity in some cases, why would plants evolve *R* genes that recognize pathogen effectors? A possible answer to this question may be illustrated in part by the host interaction between *P. syringae* DC3000 and *Arabidopsis*. *P. syringae* DC3000 actively suppresses *NHO1* expression during ETS. The expression of *NHO1* is however restored in the presence of the AvrB type III effector during ETI mediated by recognition due to the cognate R protein RPM1 (111). The induction of *NHO1* transcript by nonhost bacteria, its suppression by virulent bacteria, and the restoration of its expression during *R*-gene mediated resistance may be explained by an arms race between hosts and pathogens. Type I nonhost resistance in that case may be explained by an effective PTI, immunity which is subsequently broken down due to acquisition by pathogens of new effector genes and restored by the evolution of new *R* gene in the plant side. Type II nonhost resistance on the other hand, may also be explained by the acquisition of new effectors, possibly via

horizontal transfer from host pathogens. In this latter scenario, PTI may still be effective but the presence of the effector would also elicit ETI.

The Rxo1/avrRxo1 model of interaction

The maize *Rxo1* displays remarkable features. *Rxo1* functions to confer both host and nonhost resistances to the rice pathogen *Xoc* and the maize and sorghum pathogen *Ba*, respectively. *Ba* without *avrRba1* is virulent on *Rxo1* maize whereas *Xoc* and the bacterial blight pathogen *Xoo* are not (2). It is very likely that *Ba* is equipped with one or more effectors that enable this pathogen to interfere with PTI and cause disease whereas *Xoo* lacks such effectors effective in bringing about disease susceptibility in maize. Another important feature of the maize *Rxo1* gene is that when expressed as transgene in rice it also confers a rapid and strong HR upon challenge with *Xoc* or *Xoo* carrying the *Xoc avrRxo1* type III effector gene (3). This indicates that the necessary signaling components required to mediate nonhost resistance governed by *Rxo1* in maize are conserved in rice to confer a host resistance against rice streak disease. However, little is known about the downstream responses of rice in responses triggered by type III effectors.

My overall goal was to begin to characterize the signaling pathways triggered by the *avrRxo1/Rxo1* interactions in maize and rice. In this study, my first objective was to isolate signaling components that mediate *Rxo1*-governed resistance in cereals plants using two strategies. The first one relied on a global expression profiling of maize response to isogenic strains of *Xoo* and *Ba*, with and without the *Xoc* type III effector gene, *avrRxo1* (Chapter 2). The second strategy used a rice yeast-two-hybrid library to identify RXO1 and/or AvrRxo1 interacting proteins (Chapter 3). Finally, my second objective was to determine the function of selected signaling components, identified in the expression profiling and yeast-two-hybrid experiments, in *Rxo1*-mediated resistance using RNAi-mediated gene silencing in rice

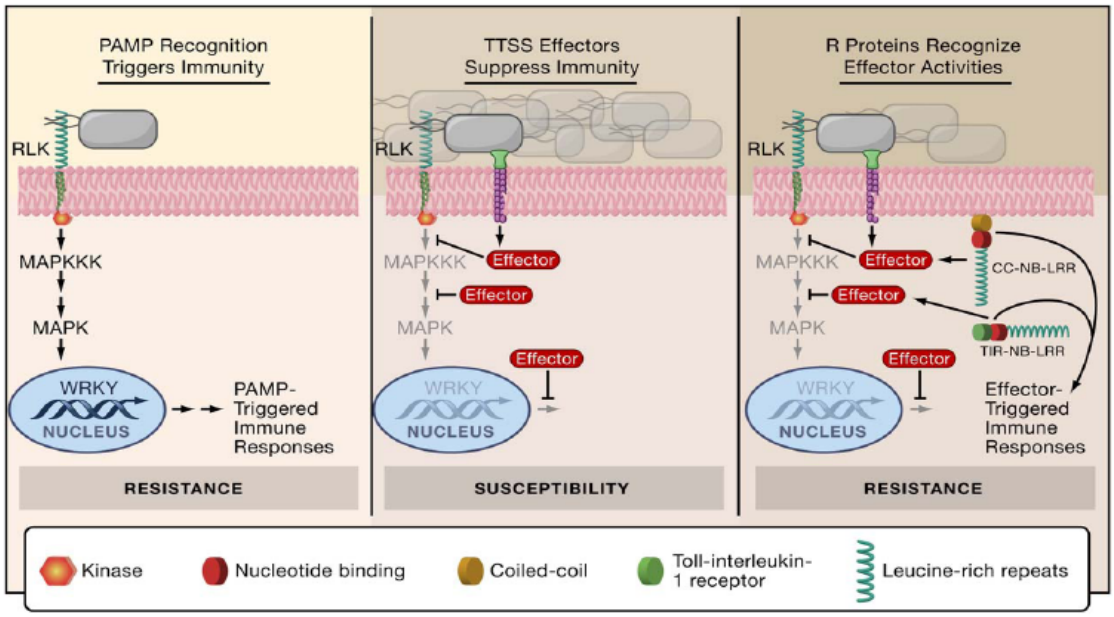

Figure 1. Model for the Evolution of Bacterial Resistance in Plants

Left to right, recognition of pathogen-associated molecular patterns (such as bacterial flagellin) by extracellular receptor-like kinases (RLKs) promptly triggers basal immunity, which requires signaling through MAP kinase cascades and transcriptional reprogramming mediated by plant WRKY transcription factors. Pathogenic bacteria use the type III secretion system to deliver effector proteins that target multiple host proteins to suppress basal immune responses, allowing significant accumulation of bacteria in the plant apoplast. Plant resistance proteins (represented by CC-NB-LRR and TIR-NB-LRR; see text) recognize effector activity and restore resistance through effector-triggered immune responses. Limited accumulation of bacteria occurs prior to effective initiation of effector-triggered immune responses.

Figure 1 Model for the Evolution of Bacterial Resistance in Plants From Chisholm et al., *Cell* 2006, 124:803-814

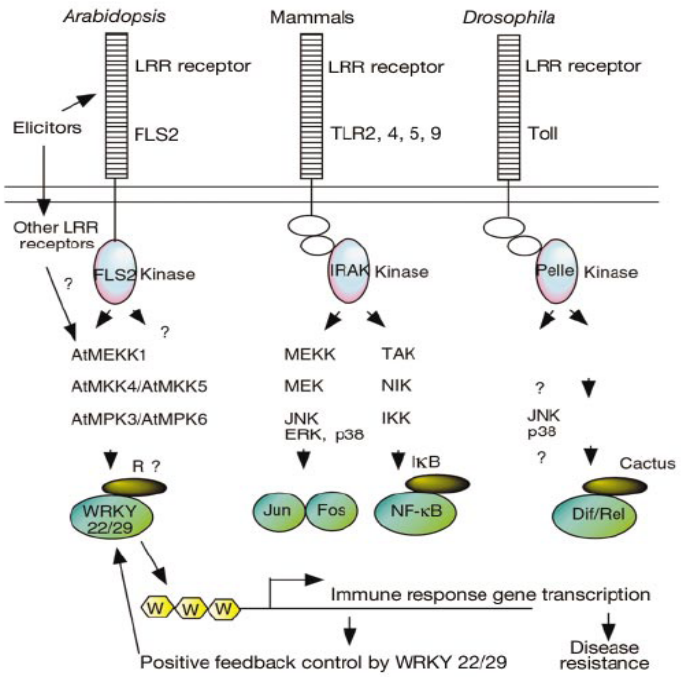

Figure 2 Model of innate immune signaling activated by LRR receptors in Arabidopsis, mammals, and Drosophila.

A putative repressor (R) could control WRKY22 and WRKY29 activity because their overexpression bypasses the requirement of elicitors. The conserved signaling pathways for innate immune responses in animals are summarized on the basis of recent reviews on mammals (2) and Drosophila (4).

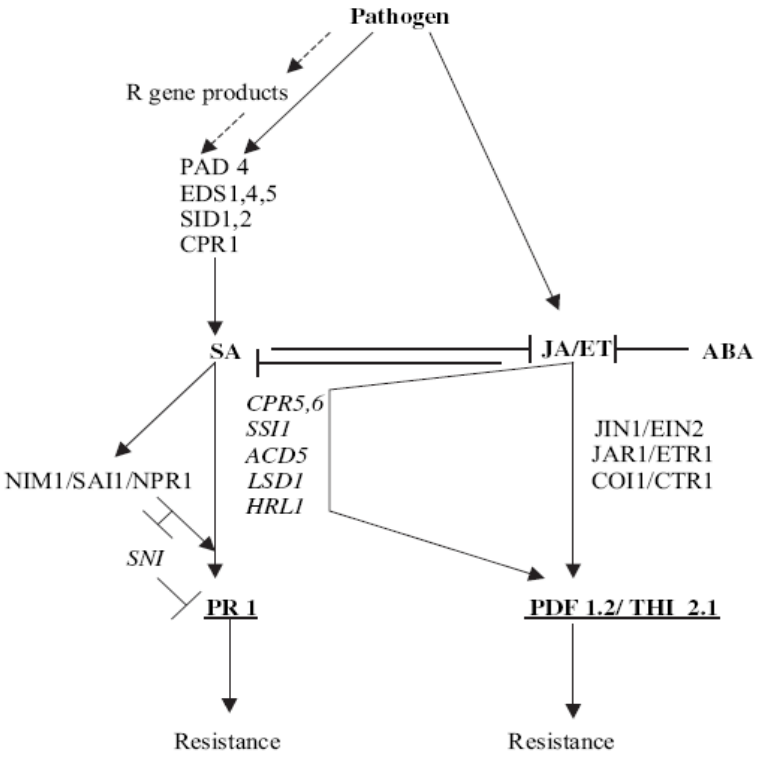

Figure 3 Model for Signaling downstream R genes. From Jalali et al, J. Phytopathology 154, 65-74 (2006)

Model showing interactions between various components of the basal defense signaling pathways Arrows indicate induction and bars indicate inhibition. *R* gene-mediated signaling superimposes on the basal defense pathway and has been shown by hashed lines. Interactions between key signaling molecules, salicylic acid (SA), jasmonate (JA), ethylene (ET) and abscisic acid (ABA) have been indicated by bold lines. Inhibitors of the defense pathways, where mutants show constitutive expression of resistance are indicated in italics. The phenotypic proteins for expression of resistance namely, PR1, PDF1.2 and THI2.1, induced by SA, JA and ET pathways respectively, have been underlined. This model is based on earlier reported models by Glazebrook (2001) and Kunkel and Brooks (2002).

Table 1 Transcription factor families that play a role in defense gene regulation

TF family	DNA-binding domain	Cis-element	Reference
WRKY	60 amino acid containg conserved sequence WRKYGQK and zinc finger motif	W-box sequence varies. Conserved core TGAC	Eulgem et al. (2000), Twick et al. (2004)
ERF	58 amino acid AP2 domain forming α-helix	GCC-box. Conserved sequence GCCGCC	Allen et al. (1998), Gutterson and Reuber

	and β-sheet DNA binding by β-sheet		(2004)
bZIP	25 amino acid region rich in basic amino acid adjacent to a leucine-rich domain 2α-helix	TGA-box. Conserved sequence TGACGTCA (core ACTG).	Meshi and Iwabuchi (1995), Fan and Dong (2002)
		ABRE-box. Conserved sequence CACGTG	Kang et al. (2002)
MYB	52 amino acid helix-turn-helix domain	Sequence varies. Conserved core TAAC	Martin and Paz-Ares (1997), Gin and martin (1999)
DOF	single zinc finger motif of C2C2 type	Sequence varies. Conserved core AAAG	Yanagisawa (2002)
Whirly	Tetrameric proteins. Conserved domain of β-sheet and α-helices bind ssDNA	Conserved sequence GTCAAA(AA)	Desveaux et al. (2004)
MYC	Basic helix-loop-helix domain	Conserved sequence CANNTG	Toledo-Ortz et al. (2003)
NAC	Twisted β-sheet surrounded by few helical elements	Conserved sequence AGGGATG	Duval et al. (2002), Ernst et al. (2004)

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CHAPTER 1 - *Xanthomonas oryzae pv. oryzae* and *Burkholderia andropogonis* carrying *Xoc-avrRxo1* co-regulate genes with different kinetics in *Rxo1*-maize and reveal putative novel cis-elements

Summary

Many maize lines carry *Rxo1*, an NB-LRR gene that confers a rapid hypersensitive response (HR) after infiltration with the rice streak pathogen *Xanthomonas oryzae* pv. *oryzicola* (*Xoc)* or the maize stripe pathogen *Burkholderia andropogonis (Ba)* carrying the effector genes *avrRxo1* or *avrRba1* respectively. To gain insights into the *Rxo1* signaling network, we used a combination of functional genomics and computational analyses. Microarray experiments were carried out to investigate the temporal expression profiles of nonhost and host responses to isogenic strains of *Xanthomonas oryzae pv. oryzae* (*Xoo*), the rice blight pathogen, and *Ba* with and without the *Xoc* type III secreted effector gene *avrRxo1*. We confirmed that *Xoc* AvrRxo1 induces disease resistance in maize when delivered by *Xoo* and showed that it also elicits the HR when delivered by *Ba*. We also show that recognition of *Xoc* AvrRxo1 induces similar transcriptional outputs, whether delivered by *Xoo* or *Ba.* Cluster analyses revealed that co-regulated genes induced after delivery of AvrRxo1 by *Xoo* or *Ba* display different kinetics and amplitudes and showed that gene clusters are associated with overrepresentation of known and putative novel DNA cis regulatory elements. The results suggest that nonhost and host resistances are very similar, only the kinetics and amplitude in the expression of genes seem to be different

Introduction

Plants have evolved two lines of inducible defense to cope with pathogen attack. PAMP (Pathogen Associated Molecular Patterns)-triggered-immunity (PTI) constitutes the first barrier effective against most pathogens and relies on the recognition through surface localized pattern recognition receptors of ubiquitous molecules present in a wide range of microbes. Successful pathogens have evolved various virulence effectors which mediate effector-triggered-susceptibility (ETS) by dampening PTI. A residual weak immune response, defined as basal

resistance (BR), remains operational during ETS but is not sufficient enough to prevent disease. The second line of defense is achieved through direct or indirect but specific recognition of a given pathogen effector by a cognate host resistance (R) protein resulting in effector-triggered-immunity (ETI). ETI is an accelerated and amplified version of BR and often culminates into a localized programmed cell death at the infection site, the so-called hypersensitive response (HR) (1). Plant defense responses involve a multitude cellular reactions including the reinforcement of cell walls, the production of antimicrobial compounds, and the upregulation of pathogenesis-related (*PR*) genes, in addition to the hypersensitive response (2-4). Phytopathogen recognition elicits an extensive temporal and spatial transcriptional reprogramming controlled by a complex signaling network consisting of the interconnected branches of PTI and ETI (5). The molecular mechanisms that translate such recognition into defined transcriptional outputs are however poorly understood.

Literature on the control of gene expression tends to focus on differential expression of genes and the function of encoded proteins and to a lesser extent on the upstream regulatory elements, or cis-elements, that modulate their expression. Cis elements are usually short and degenerate in sequence, between 4 and 12 base pairs (bp), and are responsive to transcription factors (TFs) with which they interact. In so doing, TFs can either enhance or repress target gene expression (6). DNA responsive elements govern the spatio-temporal expression of target genes and are primarily located in the upstream non-coding region of genes, although they can be located on untranslated regions as well (7). Cis elements can also group into cis-regulatory modules or units enabling the cooperative activity of different TFs (8). Understanding the complex nature of the combinatorial aspect in the regulation of gene expression remains however a major challenge.

Many maize lines carry *Rxo1*, an *NB-LRR* gene that confers a rapid HR upon challenge with the rice bacterial streak pathogen *Xanthomonas oryzae pv. oryzicola* (*Xoc*) or the maize bacterial stripe pathogen *Burkholderia andropogonis* (*Ba*) carrying the avirulence effector genes *avrRxo1* or *avrRba1* respectively (9). This remarkable feature enables *Rxo1* to function as a host or a nonhost *R* gene depending on which pathogen species maize plants are exposed to. *Ba* without *avrRba1* is virulent on *Rxo1* maize whereas *Xoc* is not virulent on maize with or without *Rxo1* (9). It is very likely that *Ba* is equipped with one or more effectors that enable this

pathogen to interfere with PTI and cause disease whereas *Xoc* lack such effectors effective in bringing about disease susceptibility.

To identify signaling components that translate AvrRxo1 recognition into defense execution, we used microarrays to monitor the global expression profiles of nonhost and host responses to isogenic strains of *Xoo* and *Ba* with and without the *Xoc avrRxo1* type III secreted effector gene in maize line B73 expressing the cognate *Rxo1* gene. Delivery of AvrRxo1 by *Xoo* or *Ba* elicited strong HR and mobilized several signal transduction pathways including pathways that have been previously associated with plant defense responses. Co-regulated genes displayed different expression kinetics. K-means clustering and cluster analyses revealed overrepresentation of the bZIP TF core recognition sequence as well as a putative novel DNA regulatory cis-element.

Results

Xoc avrRxo1 induces disease resistance when delivered by Xoo or Ba

Fourty-eight h after inoculation, *Xoo* and *Ba* carrying *avrRxo1* elicited a strong HR on maize line B73 expressing the cognate *R* gene, *Rxo1*. No HR occurred in the absence of the *avrRxo1* gene. Water soaking lesions started to appear in response to the maize pathogen *Ba* (host interaction) not expressing the *avrRxo1* gene whereas no disease symptoms appeared on plants infected with the rice pathogen *Xoo* (nonhost interaction) (Fig. 4)

The avrRxo1/Rxo1 interaction activates multiple signal transduction pathways in the course of host and nonhost responses in cereals

To gain insight into the regulatory networks mobilized by the *Rxo1/avrRxo1* interaction to bring about disease resistance, we utilized the spotted NSF-TIGR 58K maize 70-mer oligonucleotide microarray. We monitored the global expression profile of genes in response to strains of *Xoo* and *Ba* with *Xoc* avirulence effector gene *avrRxo1* as compared to their isogenic strains without the effector. Maize line B73 plants harboring *Rxo1* were challenged with the isogenic pathogens, and RNA samples harvested at 4, 8, and 24 hours post inoculation (hpi) were used for hybridization (Fig. 5).

With all time points combined, we identified a set of 767 upregulated probes in response to *Xoo (avrRxo1)* as compared to *Xoo* and a total of 646 upregulated probes in response to *Ba (avrRxo1)* as compared to the isogenic *Ba*. The encoded genes fell into several functional

categories (Fig. 6, 7). Moreover, a set of 259 probes were identified to be regulated by both host and nonhost responses; those genes were called *ARIGs* for ***a****vrRxo1/****R****xo1* **i**nduced **g**enes (Fig. 8). It is however noteworthy to mention that genes not included in the *ARIGs* may be regulated by both pathogens. This might be true if the gene was regulated by both interactions but only one made the statistical cutoff (t-test with a p-value $\leq$0.01 combined with Benjamini-Hochberg multiple testing correction) in one of the interactions or if the data was poor for one of the interactions. In contrast, no downregulated genes were observed.

Lipid signaling. Among the genes induced in the *Rxo1-avrRxo1* interactions were several genes known to be involved in the metabolism of lipid signaling compounds (Table 2). Genes encoding proteins involved in phospholipid signaling such as putative Phospholipases C, D (PLC, PLD) and PLD alpha-1 (Phosphatidylcholine-hydrolyzing phospholipase D-1) as well as inositol polyphosphate 5-phosphatase (IPP_5Pase) were upregulated as early as 8 hpi in response to *Xoo (avrRxo1).* Only PLD was induced at 24 hpi in response to *Ba (avrRxo1)* at the statistical cutoff. However, IPP_5Pase was also induced at 24 hpi in response to *Ba (avrRxo1)* at the statistical cutoff of 0.01 without multiple testing correction. PLC and PLDα-1 did not make the statistical cutoff of 0.05, or they may have been removed from the analysis due to poor data and were considered not detected (see Table 2).

Calcium network and phosphorylation dependent signaling. Several genes involved in calcium (Ca^{2+}) signaling networks were induced in response to AvrRxo1 in both pathogens. In response to *Xoo (avrRxo1)*, a putative *Ca2+ transporter* (MZ00047131) is upregulated 7 fold at 8 hpi whereas a putative *calmodulin binding protein* (*CBP*) (MZ00030850) is induced 23 fold and 15 fold at 8 and 24 hpi, respectively. These genes were not detected in the *Ba (avrRxo1)* response. In contrast, several genes encoding Ca^{2+} sensor proteins are induced in response to AvrRxo1 delivered by *Ba*. These include two putative *EF-Hand* Ca^{2+} *binding proteins* (*CCD1*) (MZ00016998 and MZ00031877), both induced as early as 4 hpi, two putative Ca^{2+} *dependent protein kinases* (*CDPKs*) (MZ00026663, MZ00057335), one induced at 4 and the second one at 24 hpi, and finally two putative *calcineurin B-like* (*CBL)-interacting protein kinase-like* (*CIPK-like*) (MZ00013618, MZ00024420) both induced at 4 and 8 hpi. Among these, one *EF-Hand* Ca^{2+} *binding protein* gene (MZ00016998), and the two CDPKs were also induced with different kinetics by *Xoo (avrRxo1)* if using a less stringent statistical cutoff. The others were not detected (Table 2).

Other upregulated protein kinase genes include *ZmPti1b* (MZ00014939) a homolog of the tomato kinase Pti that interacts with the Pto resistance gene. *ZmPti1b* and another *serine/threonine kinase* gene (MZ00039781) were both upregulated as early as 8 hpi. A homolog of rice *SAPK6* (MZ00014784), a member of the *SnRK* family of protein kinases, induced at 24 hpi. The expression of these three genes was altered in response to only *Xoo* (*avrRxo1*) at the statistical cutoff. The *serine/threonine kinase* gene (MZ00039781) was also induced by *Ba (avrRxo1)* at less stringent cutoffs.

Among the putative protein phosphatase genes induced by *Xoo* delivered AvrRxo1, six encode phosphatase 2C type of serine threonine phosphatases (PP2Cs) and two among those that are induced by *Ba (avrRxo1)* fall into the same class. All of which are not induced until 24 hpi. Below the statistical cutoff, some of these genes were induced in both interactions (Table 2). A tyrosine phosphatase gene was however induced as early as 4hpi by both pathogens.

Distinct families of transcription factors are mobilized in Rxo1-mediated resistance. Transcription factors (TFs) belonging to various gene families are upregulated during *Rxo1*-mediated resistance. Homologs of the Arabidopsis *HY5* gene and the closely related soybean *STF1* (MZ00004193, MZ00016395), both transcription factors, are induced at 4 hpi in response to delivery of AvrRxo1 by both pathogens. Maize *DBF1*, a member of the *AP2/EREBP* TF family is also induced in response to both pathogens although at different kinetics. *DBF1* is induced at 4 hpi in response to *Ba* (avrRxo1) and at 24 hpi in response to *Xoo*. Both pathogens induce expression of a gene (MZ00026127) that belongs to the *NAC* (*NAM*, *ATAF*, and *CUC*) family and homologous to rice *OsNAC4.*

At the statistical cutoff we used, several transcription factor genes were induced by delivery of *avrRxo1* only by *Xoo*, including homologs of two putative rice *homeodomain-leucine zipper* (*HD-ZIP*) transcription factor genes, *OsHox4* (MZ00027530) and *OsHox24* (MZ00010588). Both genes were induced at 4 hpi; *Hox4* expression returned to control level at 8 hpi whereas *Hox24* expression remains at similar levels from 4 to 24 hpi. Two genes (MZ00029551, MZ00056566) encoding homologs of wheat WZF1 and rice OsZF1 zinc finger proteins were similarly induced at 4 and 8 hpi in response to *Xoo (avrRxo1)*. *OsHox4*, *WZF1*, and *OsZF1* were also induced in response to *Ba (avrRxo1)* at less statistical stringency, whereas *OsHox24* was not detected.

The early induction (4hpi) of two *bZIP TFs* are observed during the interaction between maize and *Ba*-delivered AvrRxo1. The first gene (MZ00010252) is homologous to tobacco and *Arabidopsis TGA2 TFs*. The second *bZIP* TF (MZ00027914) is homologous to rice *RITA-1* gene and is upregulated at 4 and 8 hpi. Genes that are differentially regulated in response *Ba (avrRxo1)* also include homologs of rice *OsWRKY53* (MZ00020619), *OsWRKY68* (MZ00042391), and *OsWRKY76* (MZ00019246). *WRKY68* and *WRKY53* are induced early at 4hpi, but only expression of the latter is sustained at 24 hpi. *WRKY76* on the other hand is not activated before 24 hpi. Functions of *OsWRKY68* and *OsWRKY76* are unknown. *OsWRKY53*, *OsWRKY68*, and *OsWRKY76* are homologous to *Arabidopsis AtWRKY33*, *AtWRKY11*, and *AtWRKY40*, respectively. Finally, another type of transcription factor upregulated by *Ba* delivered AvrRxo1 belongs to the *R2R3-MYB* family, *Zm38* (MZ00028904); this gene is induced at 4 hpi. Below the statistical cutoff, *OsWRKY53* and *OsWRKY76* were both induced at 8 hpi in response to *Xoo (avrRxo1)*

The avrRxo1/Rxo1 interaction activates JA/ET and SA pathways. Genes encoding proteins involved in the biosynthesis of secondary signal molecules such salicylic acid (SA), ethylene (ET) and jasmonic acids (JA) were activated in response to delivery of AvrRxo1 by both bacteria. The phenylalanine ammonia lyase (*PAL*) gene is involved in salicylic acid (SA) biosynthesis and is also a central component of the phenylpropanoid pathway. Genes encoding *PAL* were upregulated at 8 and 24 hpi (MZ00025088, MZ00025089) respectively. Another gene encoding PAL is, however, was induced as early as 4hpi in response to delivery of AvrRxo1 by *Ba*. *avrRxo1* presence led to the deployment of the JA/ET pathway as indicated by the upregulation of a set of genes encoding proteins that act sequentially to generate JA (*lipoxygenases (LOX)*, *allene oxide synthase (AOS)*, *allene oxide cyclase (AOC),* and *oxo-phytodienoic acid reductase (OPDR)*) as well as the last one of the two genes involved in the biosynthesis of ET from S-adenosyl-L-methionine, the *ethylene forming enzyme* (*EFE*). *EFE* is also known as *ACC oxidase* and acts downstream of *ACC synthase*. Genes involved in JA biosynthesis were all activated at 24 hpi by both pathogen delivery of AvrRxo1 except for two LOXs which were induced as early as 4 and 8 hpi in interactions with *Xoo (avrRxo1*). *EFE* (MZ00039812) was induced at 24 hpi in response to *Ba (avrRxo1*). Two different genes (MZ00042027, MZ00057323) encoding EFE were activated by *Xoo* (*avrRxo1*) at 8 and 24 hpi respectively.

The Ubiquitin proteasome pathway. Several genes encoding components of the ubiquitin proteasome pathway were activated in response to AvrRxo1 delivered by both pathogens. These include genes coding for ubiquitination catalyzing enzymes, for structural alpha chains and catalytic beta chains that form the 20S core particle of the 26S proteasome, and for several subunits of the 19S regulatory particle. Most of these genes were upregulated at 24 hpi

Defense execution and cell death. Several genes involved in chemical and physical defenses against pathogens were coordinately activated by AvrRxo1, regardless of the pathogen delivery system. These include genes involved in the biosynthesis of flavonoid antimicrobial secondary metabolites, as well as genes involved in lignification. Expression of several *PR* genes (*PR1*, *PR5*, several protease inhibitors, etc…) as well as genes involved in the energy generating KREBS cycle and pentose phosphate pathway were also altered.

Genes involved in the regulation of cell death were upregulated in the presence of *avrRxo1* delivered by both pathogens; these include *OsBI1*, *LSD1*, *LSD1-like* (*LOL1*) *cell death suppressor lls1* (43-46).

Xoo (avrRxo1) and Ba (avrRxo1) regulated genes display different kinetics

To identify similar expression patterns, *ARIGs*, genes found to be co-regulated in response to delivery of AvrRxo1 by both *Xoo* and *Ba* at a high level of statistical significance, were clustered according to the k-means algorithm using the default setting of 5 clusters in Genespring. The average profile of gene expression indicates that delivery of AvrRxo1 by *Xoo* vs *Ba* co-regulates similar genes that display different kinetics (Fig. 9 and 10). Cluster 5 for both pathogen responses exhibits similar expression profiles. Interestingly, early expressed genes upregulated at 4 hpi in both responses and whose expression drops thereafter comprise this cluster. These genes include homologs of rice *HY5* bZIP-TF (a zinc finger transcription factor-like protein), *LOL1* which encodes another zinc finger protein similar to LSD1, and a putative *tyrosine specific protein phosphatase* among others. *Xoo* cluster 3 and 4 encode genes whose expression peaks at 8 hpi and cluster 1 and 2 genes peak at 24 hpi. All *Ba* clusters are composed of genes with expression reaching their maxima at 24 hpi except for cluster 5.

The similarity in expression patterns of the genes in some of the clusters indicates that the genes should probably be reclustered into three and four groups for Xoo and Ba interactions,

respectively. For example, the genes in cluster 1 and 2 display similar expression patterns in the *Xoo* interaction and might be considered the same cluster. This might also be true for cluster 3 and 4, and 2 and 3 in the *Xoo* and *Ba* interactions, respectively. The *ARIGs* might therefore cluster into three and four groups in response to *Xoo* (*avrRxo1*) and *Ba* (*avrRxo1*), respectively. Further reclustering will probably confirm these observations.

To confirm differences in the timing of gene expression, the profiles of four genes, two *ARIGs* (*GST7* and *ZIM*) and two genes whose expression was detected after delivery by either *Xoo* (*CBP*) or *Ba* (*PPCK*) response only, were further analyzed. In microarray analyses, *GST7* was upregulated 15 and 9 fold in response to *Xoo (avrRxo1)* at 8 and 24 hpi respectively. The same gene was only upregulated at 24 hpi in response to *Ba (avrRxo1)* with a fold change of 20. *ZIM* was however upregulated at all time points in both responses with expressions peaking at 4 (9 fold) and 8 hpi (4 fold) in response AvrRxo1 delivered by *Ba* and *Xoo,* respectively. On the other hand, a *Xoo (avrRxo1)*-regulated *CBP* was activated at 8 and 24 hpi with 23 and 15 fold, respectively and it's regulation was not statistically significant in the Ba(avrRxo1) interaction. PPCK was upregulated at 4 and 8 hpi in response to *Ba(avrRxo1)* with 23 and 9 fold changes, respectively, but was not found to be significantly upregulated in the Xoo(avrRxo1) interaction.

As shown by qRT-PCR (Fig. 11), the expression profiles of *GST7* and *ZIM* show clear differences between the two responses and strong similarity with their respective profiles observed in the microarray data, although higher fold changes were observed with qRT-PCR for most time points. In the microarray data, at the cut-off used to extablish statistical significance, the expression of *CBP* and *PPCK* was detected in response to delivery of AvrRxo1 by *Xoo* and *Ba* respectively, while in qRT-PCR activation was observed for both interactions. The expression profiles of CBP and PPCK were consistent with those observed in the microarray experiments for *Xoo* (*avrRxo1*) and *Ba* (*avrRxo1*) responses respectively. Moreover, similar to many upregulated genes, these displayed different kinetics during the two responses. It is very likely that many more than the 259 genes defined as ARIGs are regulated in response to both pathogens. These only failed to meet the stringent statistical criteria that were applied to select for differential regulation of a given gene. Statistical cutoff of 0.05 or 0.01 might reveal more overlap between the two interactions.

Gene clusters are associated with overrepresentation of known and putative novel cis-elements

Several *bZIP* and *WRKY* TFs were shown to be upregulated in response to delivery of AvrRxo1 by both *Xoo* and *Ba*. To find putative targets for these genes, we scanned promoter elements of genes comprising the different clusters. Since the rice genome is well annotated compared to maize, putative rice homologs of maize genes were retrieved with blastx and blastp using the rice genome annotation database (www.*rice.plantbiology.msu.edu*). Genes identified to have annotated homologs in rice were used to scan 1 Kb upstream of the start codon for bZIP and WRKY TFs core recognition sequences (*www.dna.affrc.go.jp/PLACE*), ACGT and TGAC. We used a hypergeometric p-value (see Methods) cutoff of less than 0.05 and a minimum threshold in which a gene in a given cluster was considered to have the motif only if it had more motifs than the average number of the motif per gene in the genome. We found that the ACGT core in *Xoo (avrRxo1)* clusters 1, 2, and 4 and in *Ba (avrRxo1*) clusters 1 through 4 occur at significantly higher frequencies than in all of the promoters in the rice genome (Table 3). The WRKY core however was not found to be overrepresented in any of the clusters. Of note, cluster 5 showed similar expression profiles in response to delivery of AvrRxo1 by both pathogens. This cluster was further analyzed with MEME (*www.meme.sdsc.edu*) in an attempt to discover conserved motifs between genes. Sequences CCCACC and CCC(A/C)CC were statistically overrepresented in genes in cluster 5 upregulated by of *Xoo(avrRxo1)* and *Ba(avrRxo1),* respectively (Fig. 12 and 13)

Discussion

The *Xoc* AvrRxo1 elicits an HR when delivered by either the rice bacterial blight pathogen, *Xoo,* or the maize stripe pathogen *Ba.* Delivery of AvrRxo1 to maize with *Rxo1* by *Xoo* and *Ba* allowed us to focus specifically on AvrRxo1 induced changes in transcription profiles by subtracting out the host- (*Ba)* and nonhost (*Xoo*) responses. Using microarrays, we reveal the multiple signal transduction pathways induced by AvrRxo1-RXO1 interactions.

Genes encoding proteins involved in lipid signaling were shown to be rapidly induced. These include *PLC*, *PLD*, and *IPP$_5$PPase* which likely act in concert. PLC is indeed known to hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP2) into two secondary messengers, inositol 1,4,5-trisphosphate (IP3), which diffuses into the cytosol, and diacylglycerol (DAG), which

remains in the membrane (10). DAG is rapidly phosphorylated in plants and converted into phosphatidic acid (PA) (11). PA has been shown to trigger the oxidative burst in *Arabidopsis* and to activate a MAP kinase cascade in soybean (12, 13). PA can also be generated through direct activity of PLD which produces choline and PA from structural phospholipid precursors such as phosphatidyl-choline (PC) (14). PLD was shown to be recruited to the plasma membrane of rice cells in response to *Xoo* (15). Recent studies have indicated that elicitor induced reactive oxygen species (ROS) generation occurs in a biphasic manner in rice cell suspension culture and that both PLC and PLD are activated during the first transient burst. However, only PLD activity was sustained during the second burst, and this activation was associated with its recruitment to the plasma membrane (16). The early expression of both PLC and PLD and the sustained expression of PLD at 24 hpi is consistent with the observation described at the protein level in rice.

Several protein kinases and phosphatases were induced both early and late in response to AvrRxo1. Protein kinase genes include *CIPKs*, *CDPKs*, and other protein kinases such as a homolog of rice *SAPK6* induced at 24 hpi. Expression of *SAPK6* was shown to be activated in rice in response to hyperosmotic stress and abscisic acid hormone (ABA). Moreover, *in vitro* kinase assays showed that SAPK6 is able to phosphorylate itself and to hyperphosphorylate members of the bZIP family of transcription factors that interact with ABA-responsive elements (ABRE). Yeast-two-hybrid assays also showed that SAPK6 interacts with rice OREB1, a member of the bZIP family of transcription factors (17). Several PP2C genes were induced at 24 hpi. Some PP2Cs have been implicated in ABA responses (18-22), but their targets are unknown. An *Arabidopsis* PP2C, AP2C1 was however shown to inactivate stress-activated MAP kinases (MPKs), MPK4 and MPK6, to negatively regulate JA and ET biosynthesis and to compromise innate immunity against the necrotrophic pathogen *Botrytis cinerea* (23). This suggests that PP2Cs might regulate MPK signaling cascades and defense responses. Taken together, these data suggest that the ABA pathway may play an important role in *Rxo1*-mediated resistance and/or that these genes are regulated via another pathway. It is very likely however that SAPK6 and some of these PP2C function in connection and possibly in a MAPK cascade leading to activation of members of the bZIP family of TFs.

Distinct classes of TFs were induced in response to AvrRxo1. Homologs of the rice *HY5* and *OsNAC4* TFs, as well as maize *DBF1* were induced in response to both pathogens. *HY5* is also homologous to the closely related soybean *STF1* and *Arabidopsis HY5* genes. *AtHY5*

belongs to the basic leucine zipper (bZIP) family that recognizes core ACGT motifs and is a positive regulator of photomorphogenesis (24). AtHY5 forms a heterocomplex with a closely related protein of the same family (25). STF1 is known to form homodimers and to recognize the sequences containing the TGACGT core sequence (26). *DBF1* was shown to be upregulated in response to ABA and drought stress and its product recognizes an ACCGAC core sequence in the promoter of the ABA-inducible *rab17* gene in maize (27). Although a very limited number of *NAC* genes have been characterized, yeast-one-hybrid assays have shown that distinct groups of NAC TF can bind the same NAC core DNA binding site, the CATGTG sequence (28, 29).

Transcription factors that are induced in response to *Xoo*-delivered AvrRxo1 include homologs of rice *OsHox4* and *OsHox24* and two zinc finger protein encoding genes homologous to wheat *WZF1* and rice *OsZF1*. *OsHox4* and *OsHox24* belong to family I of HD-ZIP putative TFs that interact with similar pseudopalindromic DNA (CAATNATTG) sequences as homo or heterodimers between members of the same family. Family I members show preference to CAAT(A/T)ATTG in *Arabidopsis* (30). The expression of *HD-Zip I* members in *Arabidopsis* was shown to be regulated by abiotic stresses and by hormones such as abscisic acid (ABA) and ET. Overexpression of *OsHox4* in rice resulted in semi-dwarf phenotype and impaired gibberellin (GA) signaling whereas downregulation of this gene showed no obvious phenotype (31). Given the observation that homologs of *OsHox4* and *OsHox24* are both upregulated as early as 4 hpi, it is possible that the encoded proteins function in a heterocomplex that fine tunes the response to AvrRxo1 recognition. On the other hand, WZF1 was shown to interact with a CATCCAACG nonamer-containing 38 base pair DNA fragment present in the promoters of wheat histone genes 3 and 4 (32). We did indeed observe the upregulation WZF1 homolog at 4 hpi and 8 hpi, followed by the activation of three probes encoding histone 3, two other probes encoding histone 1-like proteins, one probe encoding histone H2B, as well as several others involved in chromatin remodeling in response to *Xoo*-delivered AvrRxo1. It is possible that these genes represent transcriptional targets for these TFs.

Members of the *bZIP*, *WRKY*, and *R2R3-MYB* families of TFs were found to be induced early during the maize response to *Ba (avrRxo1)*. These include two *bZIP TFs*, one homologous to tobacco and *Arabidopsis TGA2 TFs*, and the other to the rice *RITA-1* gene. TGA2 TFs form homo and heterodimers between members of the same family and with the ankyrin-repeat protein NPR1, a central activator of SA-mediated expression of *PR* genes (33). TGA2 TFs

recognize the consensus *as-1* sequence consisting of two repeated TGACGTCA palindrome spaced by 12 nucleotides (33). The second bZIP TF, *RITA-1* was shown to bind the palindromic elements TACGTA, GACGTC, and CACGTG, all having the ACGT core sequence and to be involved in seed development (35). The two encoded proteins may also work in concert to regulate plant immune responses. Homologs of rice *OsWRKY68* and *OsWRKY53* were induced early at 4 hpi in response to *Ba*-delivered AvrRxo1, but only expression of the latter was sustained at 24 hpi, whereas a homolog of *OsWRKY76* was activated at 24 hpi. *OsWRKY53* was shown to be induced in rice cultured cells as well as in plants by fungal elicitors and by the blast fungus *Magnaporthe oryzae* respectively. *OsWRKY53* was also shown to bind W-box elements with the TGAC core sequence, to activate expression of *PR* genes, and to confer enhanced resistance to *M. oryzae* when overexpressed in transgenic rice plants (36). Functions of *OsWRKY68* and *OsWRKY76* are unknown. These two genes are, however, homologous to *Arabidopsis AtWRKY11* and *AtWRKY40,* respectively, whose functions are known. OsWRKY53 is homologous to AtWRKY33 which was shown to exist in a nuclear complex that includes MPK4 and MSK1 in the absence of stimuli. Challenge with *Pseudomonas syringae* or flagellin, led to the activation of MPK4, phosphorylation of MSK1, and the release of WRKY33 from the complex. Moreover, AtWRKY33 activates the expression of *PAD3* involved in the biosynthesis of phytoalexin antimicrobial compounds (37). *AtWRKY11*, on the other hand, is similarly induced in response to virulent and avirulent strains of *P. syringae* and acts as a negative regulator of BR in *Arabidopsis* (38). *AtWRKY40* also is induced by virulent and avirulent strains of *P. syringae* and by the necrotrophic fungal pathogen *B. cinerea* (39, 40). Moreover, AtWRKY40 interacted with itself and with AtWRKY18 and AtWRKY60 in yeast-two-hybrid assays. While single knockout mutants of these genes showed little or no phenotype with respect to defense, *Atwrky40*/*Atwrky18* exhibited enhanced resistance to *P. syringae* but increased susceptibility to the *B. cinerea* fungus (39). These three WRKY TFs may work in an interacting genetic and/or biochemical complex to mediate responses to plant pathogens. *Zm38* belonging to the *R2R3-MYB* family of TFs was also induced early in the interaction with *Ba (avrRxo1)*. Zm38 was shown to negatively regulate the biosynthesis of anthocyanin flavonoids in maize (41), whereas C1 and Sn, an R2R3-MYB and a bHLH factor respectively, were shown to cooperate in positively regulating the synthesis of these secondary metabolites (42). MYB recognition elements were also shown to form light regulatory units with bZIP recognition elements. Both

recognition elements were necessary for light induced activation of several genes involved in the biosynthesis of flavonol flavonoids (43). Accordingly, several genes involved in flavonol biosynthesis were found to be upregulated including *PAL*, *C4H* (*Cinnamate-4-hydroxylase*), *CHS* (*Chalcone synthase*), and *F3H* (*Flavanone-3-hydroxylase*).

Signal transduction pathways previously implicated in defense against pathogens were elicited by the avrRxo1/Rxo1 interaction. Genes encoding proteins implicated in the SA and JA/ET signal transduction pathways were induced in response both pathogens.

Genes involved in the regulation of cell death are upregulated in the presence of *avrRxo1*; these include negative regulator of cell death such as *OsBI1*, *LSD1*, and the cell death suppressor *lls1* (44-46) as well as a positive regulator of cell death, *LSD1-like* (*LOL1*) (47). LOL1 may promote the hypersensitive cell death whereas OsBI1, LSD1 and lls1 may confine the spread of cell death to infection sites

The ACGT core recognized by TFs of the bZIP family is overrepresented in most clusters. Several bZIP TFs are upregulated in response to delivery of AvrRxo1 by both pathogens and they likely target some of these upregulated genes, possibly in cooperation with other TFs. So long as their binding sites are known, scanning the promoters of *Xoo* and/or *Ba* regulated genes can point to putative targets for the different TF families determined to be activated in response to these bacteria. Using a less targeted approach, we attempted to find conserved motifs in upstream regulated genes using MEME (48, 49). We found two similar motifs (CCCACC and CCC(A/C)CC) that were overrepresented in both *Xoo (avrRxo1)* and *Ba (avrRxo1)* cluster 5. These may represent core recognition sites for as yet unidentified upstream TFs that regulate the expression of these genes. A yeast one hybrid assay using promoters of some of these genes should help in that direction.

Several genes involved in signal transduction were found to be regulated in response to delivery of AvrRxo1 by *Xoo* and *Ba*, including genes encoding lipid and calcium signaling molecules, protein kinases and phosphatases, as well as TFs. Some of these genes have been described earlier in relation to biotic and/or abiotic stresses and development, while others have not. Data generated in this work provides a platform for generating rationale based hypotheses that amend functional investigations into the molecular bases that govern plant innate immunity.

Material and methods

Plant material and treatments

Seeds from maize line B73 were cleansed of potential fungal contamination by soaking in distilled water for 4 hours, and then heating at 65^{o}C for 10 minutes in a water bath. Seeds were then sterilized with 10% bleach for 5 minutes, rinsed with sterile water and blotted dry with sterile paper towels before culturing on water agar for 4 days. Germinating seeds were sown in flats in a 10 cm-deep soil: peat: perlite mix (2:1:1). Seedlings were grown in greenhouses under 12 hr light/ 30°C and 12 hr dark/ 25°C. The 4th fully expanded leaves of three-week-old seedlings were used for bacterial infiltration with a needle-less syringe. Leaves were harvested at 4, 8 and 24 hours after inoculation, pooled and frozen in liquid nitrogen then stored at -80°C until ready for use. Plants were examined 24 and 48 hours for HR, no HR or water soaking.

Bacterial strains and culture media

Xanthomonas oryzae pv. *oryzae* and *B. andropogoni*s with and without *avrRxo1* were infiltrated into leaves of maize line B73 to determine the global expression signature in response to these bacteria. *Xanthomonads* were grown in nutrient broth at 28^{o} C. Strains of *B. andropogonis* were grown in PS broth at 28 ^{o}C. The antibiotic spectinoymcin (100 ug/ul) was added to culture media to maintain selection of the plasmid harboring *avrRxo1*. Bacterial cultures were centrifuged (5,000 rpm for 6 min), and the pellets resuspended in distilled water. Cell density was adjusted to 5x10^{7} cfu/ml and infiltrated into seedling leaves. The *Xoo* strain has been previously described (9). The *Ba* strain was made the same. Both strains were transformed with the pHM1 vector carrying avrRxo1 with its native promoter.

Microarray analysis

Total RNA was isolated using Trizol^{TM} (Life Technologies, Rockville MD) reagent following the manufacturer's recommendations. Fifty micrograms of total RNA from each sample was used for cDNA synthesis and labeled with Cy3 and Cy5 dyes using a Genisphere Array 50 Kit according to the manufacturer's protocol (Genisphere Inc.PA) and hybridized to NSF TIGR 58K maize oligonucleotide microarrays (http://www.maizearray.org/, AZ). Slides were scanned using Genepix-pro 6.0. All subsequent data analyses were performed using Genepring GX7.3 (Agilent technology). Datasets were normalized according to Lowess. We

applied cutoffs of two-fold differential regulation, and a t-test with a p-value of 0.01 combined with Benjamini-Hochberg multiple testing correction. Default settings of k=5 were used to generate all clusters.

Quantitative RT-PCR analyses

The same RNA samples used for microarray analyses were used for real-time PCR. RNA samples were subjected to DNAse I (Promega Madison, WI) treatment and 10ug of total RNA were reverse transcribed using superscript III reverse transcriptase from Invitrogen (Carlsbad, CA) according to manufacturer protocols. cDNA derived from 100 ng of total RNA was used for qRT-PCR with the Bio-Rad iCycler iQ using SYBR green Supermix (VWR West Chester, PA). Three biological replications were performed and the fold change of gene expression for all was determined using the ΔΔCt method. Rice 18S primers were used as internal control for *Xoo* treatments and the *Rxo1* gene was used as a control for *Ba* treatments because the 18S showed variability in Ba responses. Primers used in this study are listed in a table of primers (Table 6, chapter 3).

Motif identification

All 259 probes that comprise the ARIGs were blasted against the maize genome database (TIGR, maize AZM_5) to recover sequences from which they were designed. These were compared to the rice genome database (Rice Genome Annotation Project: www.*rice.plantbiology.msu.edu*) to recover most homologous rice genes for each maize sequence based on the amino-acid sequence conservation genes by using blastx and blastp. 1Kb upstream sequences from the start codon were extracted to scan for known motifs and to discover putative novel motifs using PLACE (*www.dna.affrc.go.jp/PLACE*) and MEME (*www.meme.sdsc.edu*) respectively. The statistical significance of motif overrepresentation was assessed using the hypergeometric test below:

$$p = P(i \geq h) = \sum_{i=h}^{\min(C,H)} \frac{\binom{H}{i}\binom{G-H}{C-i}}{\binom{G}{C}}$$

Where G is the total number of genes in the genome, C is the number of genes in the cluster, H is the number of hits in all genes and h is the number of hits in the cluster. We considered a gene to be a hit only if it had equal or more motif number than the average number of the motif within the genome. The hypergeometric score has proven to be a robust test for assessing the overrepresentation of cis-elements (50).

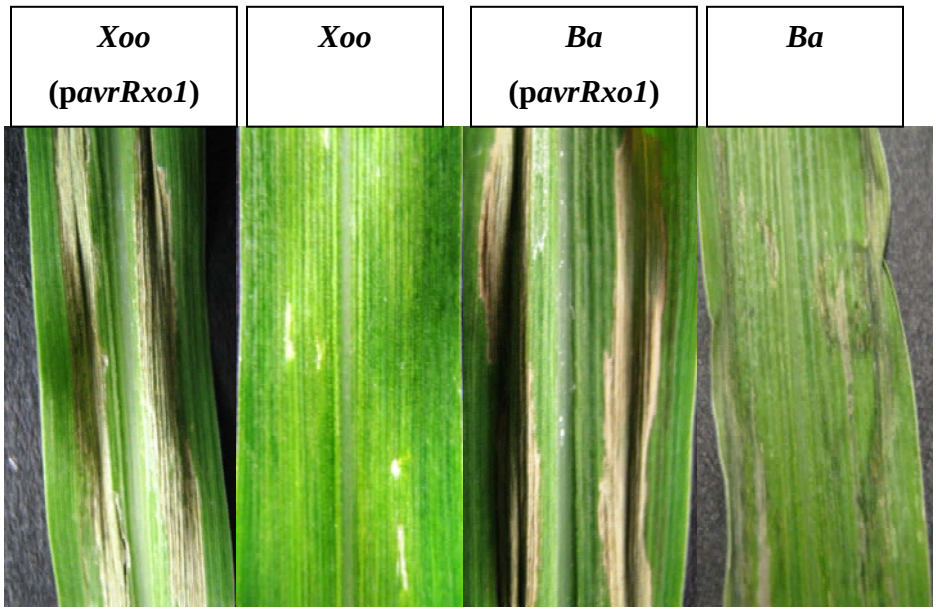

Figure 4 Phenotypic responses to *Xoo* and *Ba* strains

Maize lines B73 harboring Rxo1 were inoculated with *Xoo* and *Ba* with and without the pHM1 plasmid carrying the *avrRxo1* gene under the control of its native promoter. Pictures were taken 48 hours post inoculation. Bacteria expressing *avrRxo1* elicit the HR whereas *Xoo* does not and *Ba* causes water soaking

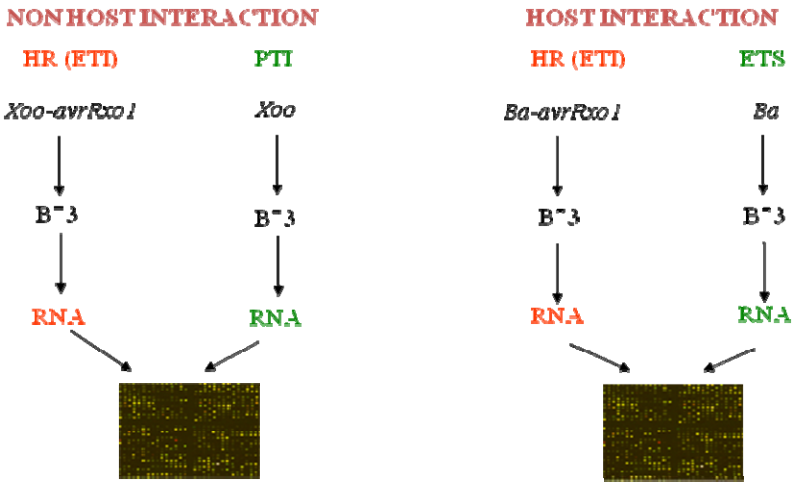

Figure 5 Microarray experimental design

RNA samples were isolated 4, 8 and 24 hpi with the bacteria and labeled cDNAs were hybridized to the chips. 4 biological replications and 2 dye swaps were performed for each time point. 2-fold differential regulation and p-value≤0.01 with multiple testing corrections were considered differentially regulated

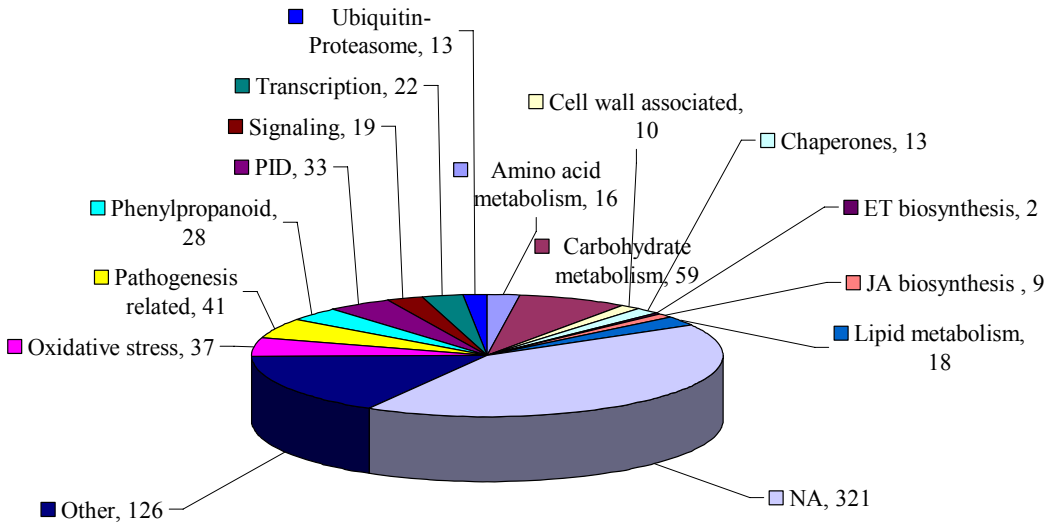

Fig. 3A. Functionnal distribution of *Xoo-avrRxo1* upregulated genes

Figure 6 Functional distribution of *Xoo-avrRxo1* upregulated genes

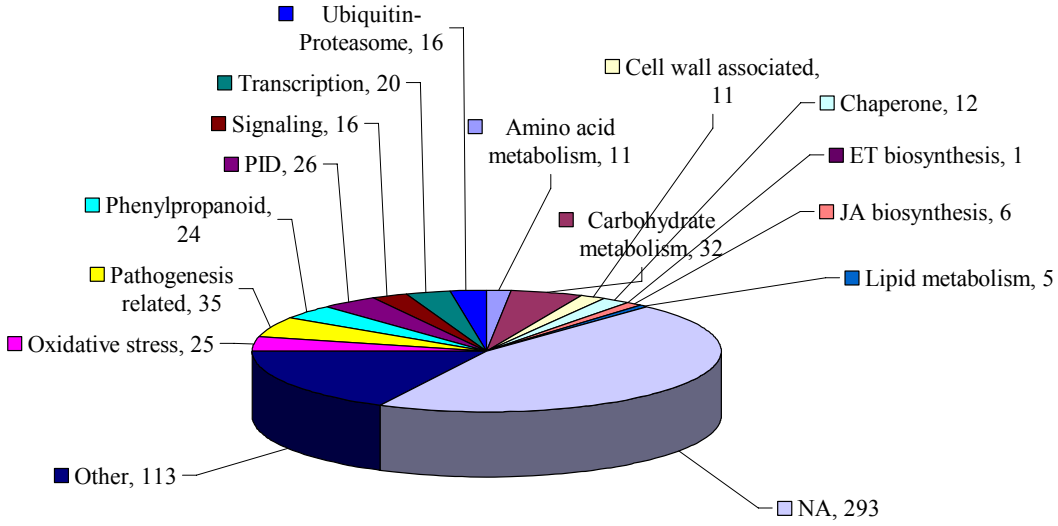

Fig. 3B. Functionnal distribution of *Ba-avrRxo1* upreguled genes

Figure 7 Functional distribution of *Ba-avrRxo1* upregulated genes

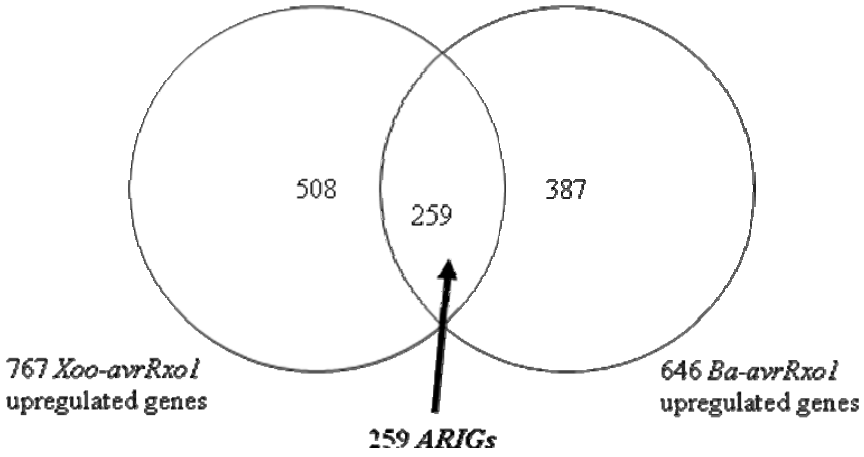

Figure 8 *ARIGs: avrRxo1/Rxo1* induced genes.

Genes upregulated at all time points shows that 259 probes overlap between *Xoo* and *Ba* responses at high statistical cutoff

Figure 9 Profiles of the 259 *ARIGs* in response to *Xoo* (*avrRxo1*)

The average value of fold change for each time point was calculated and plotted to generate the average profile of each cluster

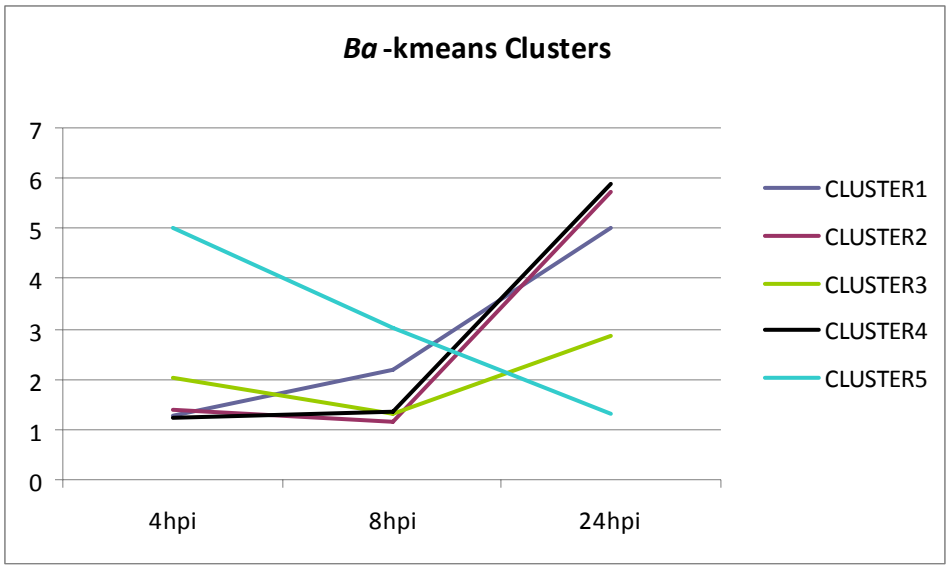

Figure 10 Profiles of the 259 *ARIGs* in response to *Ba* (*avrRxo1*)

The average value of fold change for each time point was calculated and plotted to generate the average profile of each cluster

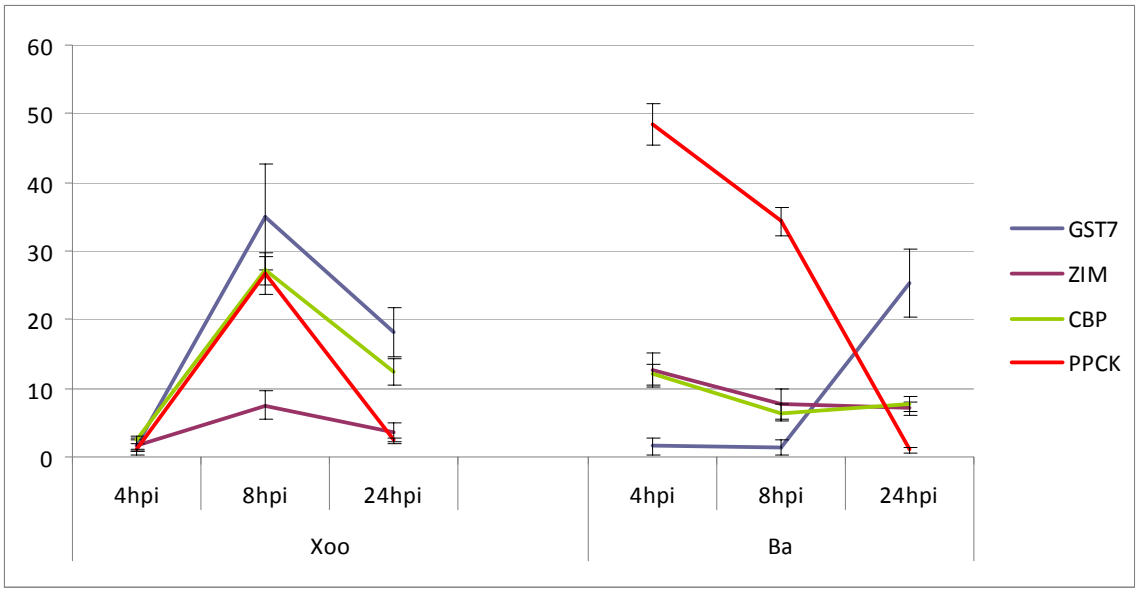

Figure 11 Quantitative real-time PCR shows Xoo and Ba co-regulate genes with different kinetics and amplitudes

qRT-PCR was carried out using three biological replications. Maize gene specific primers were used along with rice 18S primers as internal controls for *Xoo*. *Rxo1* primers were used as internal controls for Ba responses. Fold changes were calculated using the ΔΔCt method.

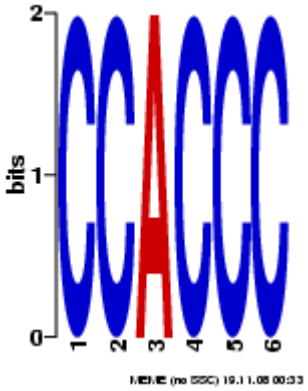

Figure 12 *Xoo5* sequence logo

Xoo	Hypergeometric p-value	Percentage of hits (having 1 or more CCACCC)	Average number of cis elements
Cluster 5	$2.9E^{-03}$	83%	1.33
Genome		40%	0.46

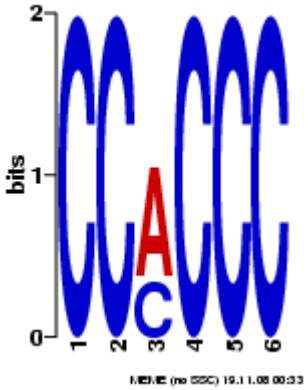

Figure 13 *Ba5* sequence logo

Ba	Hypergeometric p-value	Percentage of hits (having 1 or more CC(A/C)CCC)	Average number of cis elements
Cluster 5	$4.1E^{-02}$	73%	2
Genome		54%	1.06

Sequence logos for conserved motifs overrepresented in early induced genes in cluster 5 and associated statistical analyses

The logos indicate position weighing matrices for each nucleotide in the motif, where "2" represents presence of the nucleotide in all sequences of the training set used by MEME to generate the motif. The tables below logos indicate the representation of the motifs in the genome and the clusters and associated hypergeometric p-values

Table 2 Subset of genes induced in the *Rxo1* interactions that are highly significant for at least one treatment at any time point

(p-value, *≤0.05; **≤0.01; ***significance at <0.01 with multiple testing correction; ND: not detected, these may fall below the cutoff p-value ≤0.05 or simply not included due to poor data. Genes that are shown in the table fall in functional categories (mainly signal transduction genes) that were emphasized in the text. Genes in blue and yellow shadings were significantly induced, designated as "***", in the *Xoo* and *Ba* interactions, respectively; genes shown in red were significantly induced in both interactions.

Probe ID number	4 hpi	8 hpi	24 hpi	**Predicted protein**	4 hpi	8 hpi	24 hpi
	Xoo interaction				**Ba interaction**		
MZ00026245	1.4	2.4***	4.3***	*PLC*	ND	ND	ND
MZ00005530	0.8	3.0***	2.4***	*PLD*	0.9	1.4	3.3***
MZ00024625	0.8	0.9	2.2***	*PLDα-1*	ND	ND	ND
MZ00036624	0.7	2.7***	4.6***	*IPP$_5$Pase*	1.2	0.9	3.0**
MZ00047131	0.8	7.7***	1.5	*Ca^{2+} TRA*	ND	ND	ND
MZ00030850	0.8	23.0***	15.1***	*CBP*	ND	ND	ND
MZ00016998	1.1	5.2**	1.1	*EF-Hand*	3.1***	1.7	4.7***
MZ00031877	ND	ND	ND	*EF-Hand*	3.4***	1.1	1.2
MZ00026663	1.8	1.0	2.0**	*CDPK*	2.1***	1.8	1.3
MZ00057335	0.9	2.4**	1.1	*CDPK*	1.0	1.2	2.1***
MZ00013618	ND	ND	ND	*CIPK-Like*	2.3***	2.1***	1.1
MZ00024420	ND	ND	ND	*CIPK-Like*	5.3***	4.0***	0.6
MZ00014939	0.8	3.6***	0.9	*Pti1b*	ND	ND	ND
MZ00039781	0.8	5.1***	2.1***	*PK*	2.0**	2.14*	1.6
MZ00014784	0.8	0.8	2.8***	*SAPK6*	ND	ND	ND
MZ00016817	0.7	1.0	2.6***	*PP2C*	0.9	1.0	2.4**
MZ00020299	1.7	1.0	5.0***	*PP2C*	3.4**	3.0**	1.5*
MZ00026155	0.4	0.8	2.3***	*PP2C*	ND	ND	ND
MZ00028000	0.9	0.9	3.6***	*PP2C*	0.8	2.5**	2.0**

MZ00046397	0.9	0.9	3.1***	*PP2C*	ND	ND	ND
MZ00046421	0.6	0.7	2.4***	*PP2C*	ND	ND	ND
MZ00018283	ND	ND	ND	*PP2C*	1.4	1.2	2.6***
MZ00019552	ND	ND	ND	*PP2C*	1.3	1.3	3.0***
MZ00015576	3.4***	1.4	0.5	*Tyr-PPase*	3.7***	2.3***	0.6
MZ00004193	6.9***	2.8***	0.7	*HY5*	4.1***	2.4***	0.9
MZ00016395	2.4**	1.6	0.7	*STF1*	2.3***	1.7	0.6
MZ00013205	ND	ND	ND	*DBF1*	3.5***	2.3***	0.5
MZ00015673	1.7	1.5	2.4***	*DBF1*	0.3**	3.8**	1.5**
MZ00026127	1	1.3	2.1***	*NAC*	1.4	1.4	2.9
MZ00027530	2.4***	1.2	0.6	*OsHox4*	0.4**	1.7	0.5**
MZ00010588	3.4***	2.0	3.2***	*OsHox24*	ND	ND	ND
MZ00029551	2.8***	3.9***	1.0	*WZF1*	4.5**	2.6	0.7
MZ00056566	2.4***	3.0***	0.6	*OsZF1*	0.3**	2.3**	0.8
MZ00010252	ND	ND	ND	*TGA2*	2.4***	1.7	1.2
MZ00027914	ND	ND	ND	*RITA-1*	2.7***	2.1***	0.8
MZ00020619	1.1	2.3**	0.7	*OsWRKY53*	2.3***	1.7	2.3***
MZ00042391	ND	ND	ND	*OsWRKY68*	2.1***	1.3	0.8
MZ00019246	1.0	6.4**	1.2	*OsWRKY76*	0.7	0.3	7.5***
MZ00028904	ND	ND	ND	*Zm38*	2.3***	1.2	0.5
MZ00025088	1.6	2.4***	1.5	*iPAL*	0.5**	1.7**	1.4**
MZ00025089	1.5	2.6**	1.5	*iPAL*	1.9	1.8	2.1***
MZ00049296	ND	ND	ND	*PAL*	2.7***	1.6	0.8
MZ00000026	4.1***	4.9***	0.7	*LOX*	4.5**	3.2**	0.6
MZ00000666	1.0	7.3***	0.3	*LOX*	ND	ND	ND
MZ00000521	1.2	3.1**	1.3	*LOX*	0.9	0.9	6.8***
MZ00013846	ND	ND	ND	*LOX*	1.4	1.3	2.2***
MZ00044190	0.9	1.6	6.8***	*AOS*	1.7	1.4	6.3***
MZ00043517	1.1	0.9	2.2***	*AOC*	1.6	1.1	3.7***
MZ00056579	0.7	1.0	5.4***	*OPDR*	0.7	0.5	10.3***
MZ00042027	0.8	3.6***	1.6	*EFE*	ND	ND	ND
MZ00057323	1.1	1.1	3.0***	*EFE*	ND	ND	ND
MZ00039812	0.7	2.8**	1.7	*EFE*	0.7	0.8	2.5***
MZ00043996	0.8	6.1***	1.2	*OsBI1*	3.2***	1.8	2.5***
MZ00041803	6.7***	1.8	0.5	*LSD1*	0.07**	2.6**	0.5**
MZ00041804	3.5***	1.7	0.5	*LSD1*	ND	ND	ND
MZ00041802	3.9***	1.7	0.5	*LOL1*	10.1***	2.6***	0.5
MZ00027113	1.1	0.9	4.7***	*lls1*	1.3	1.4	3.4***
MZ00026477	ND	ND	ND	*PPCK*	23.4***	8.6***	0.8
MZ00017926	1.4	14.7***	9.3***	*GST7*	0.8	1.9	20***
MZ00057056	2.6***	4.0***	2.0***	*ZIM*	8.9***	5.5***	4.8***
MZ00002477	1.7	2.0***	3.0***	*H3*	ND	ND	ND
MZ00023382	1	1.1	3.5***	*H3*	ND	ND	ND
MZ00039174	1.4	0.8	2.8***	*H3*	ND	ND	ND
MZ00023714	0.4	0.4	2.5***	*H1-LIKE*	ND	ND	ND
MZ00034952	0.5	0.4	2.1***	*H1-LIKE*	ND	ND	ND
MZ00013391	2.5***	1.2	0.9	*H2B.3*	4.3**	1.4**	0.9
MZ00043784	1.3	1.6	2.2***	*C4H*	1.2	1.3	2.3***
MZ00041915	1.0	1.0	2.6***	*CHS*	1.1	0.9	2.1***
MZ00027767	7.2***	1.8	7.7***	*F3H*	2.5***	1.0	7.6***

Table 3 Statistical analyses for the overrepresentation of the ACGT core in *Xoo* and *Ba* clusters

Xoo	**Hypergeometric p-value**	**Percentage of hits (having 3 or more ACGT)**	**Average number of cis elements**
Cluster 1	$3E^{-07}$	73%	5.57
Cluster 2	$3.9E^{-10}$	73%	4.98
Cluster 3	0.2	30%	2.80
Cluster 4	$5.6E^{-04}$	80%	5.33
Cluster 5	0.2	42%	3.66
Genome		**36%**	**2.16**
Ba	**Hypergeometric p-value**	**Percentage of hits (having 3 or more ACGT)**	**Average number of cis elements**
Cluster 1	$4.7E^{-06}$	100%	6.16
Cluster 2	$1.2E^{-07}$	70%	4.80
Cluster 3	0.01	61%	4.77
Cluster 4	$1.0E^{-05}$	68%	6.18
Cluster 5	0.06	50%	4.00
Genome		**36%**	**2.16**

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CHAPTER 2 - A conserved protein kinase and a master transcriptional regulator that interacts with RXO1 mediate the hypersensitive response elicited by AvrRxo1 in rice

Summary

The maize *Rxo1* gene encodes an NB-LRR protein that confers a hypersensitive response (HR) against the rice bacterial leaf streak pathogen, *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*) expressing the *avrRxo1* type III effector gene. This recognition function of *Rxo1* occurs in maize or when the gene is expressed as a transgene in rice. Gene expression analyses of *Rxo1* maize plants after inoculation with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) expressing the *Xoc avrRxo1* identified the early induction of a tomato ortholog of the *Pto interacting 1* (*Pti1*) gene encoding a serine threonine kinase, *ZmPti1b*. Using a full length coding sequence as bait to screen a yeast-two-hybrid library, we identified 11 rice proteins that interact with RXO1. Here, we describe the functional analysis of the putative rice ortholog of *ZmPti1b*, *OsPti1a*, and two genes encoding RXO1 interacting proteins, *Os1PVOZ* and *OsATL6*. RNAi-mediated gene silencing reveals that *OsPti1a*, a serine threonine kinase gene, and *Os1PVOZ*, encoding a putative transcription factor, are required for *Rxo1*-dependent HR whereas *OsATL6*, encoding a putative RING finger type E3 ubiquitin ligase is dispensable. Computational analysis aimed at identifying DNA responsive elements within the promoter of all rice genes, suggests that Os1PVOZ is a master regulator of many signal transduction pathways, including those that mediate plant innate immunity. These results suggest that Rxo1 activation is mediated by phosphorylation and early extensive transcriptional cascades that culminate into the hypersensitive response.

Introduction

A major objective in field of host microbe interactions is to understand the molecular mechanisms by which plants recognize pathogens and trigger resistance. Disease resistance (*R*) genes are key components of the plant surveillance system. Specific recognition of a pathogen avirulence (avr) effector by the corresponding R protein often elicits rapid programmed cell

death (PCD) termed the hypersensitive response (HR); this response may restrict subsequent pathogen invasion (1-3). The majority of *R* genes encode predicted cytoplasmic nucleotide-binding-leucine-rich repeat (NB-LRR) proteins. Prevailing models for R protein function indicate that they are kept inactive through intra and inter-molecular interactions and are activated following modifications inflicted by pathogen virulence effectors on their associated partners or through direct binding to NB-LRR proteins (4). This activation is thought to be achieved through conformational change and the exchange of ADP for ATP enabling the exposure of binding platforms for yet to be identified downstream signaling components (4). Activation of R proteins by pathogen effectors invokes massive changes in the expression of genes following defined spatial and temporal programs. Signal transduction and amplification are often achieved through posttranslational modifications of preexisting signal molecules including protein kinases and phosphatases, molecules involved in protein turnover, and transcription factors which mediate reprogramming of cellular functions.

Compelling evidence implicates kinase signaling cascades in effector triggered immunity (ETI). The rice XA21 R protein for instance is a receptor-like kinase (RLK) that allows specific recognition of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) in a gene for gene manner (5). Likewise, the tomato *Pto* gene encodes a serine threonine kinase that mediates resistance elicited by *P. syringae* AvrPto and AvrPtoB effectors (6). However, their downstream targets in plant cells remain to be determined. Pto interacts with a second serine threonine kinase, Pti1, in yeast-two-hybrid assays. Moreover, Pti1 is phosphorylated by Pto in vitro and its overexpession accelerates the HR in response to *Pseudomonas syringae* expressing *avrPto* (7). Mitogen-activated kinases (MPKs) WIPK (wound induced protein kinase) and SIPK (salicylic acid induced protein kinase) were shown to be rapidly activated in response to both tobacco mosaic virus and Avr9 treatment in tobacco plants expressing *N* and tomato *Cf9* R genes respectively (8, 9). *Arabidopsis* orthologs of *WIPK* and *SIPK*, *MPK3* and *MPK6* respectively, have been implicated in PAMP-triggered-immunity (10).

Recent studies have shown that some R proteins, upon activation by the corresponding Avr proteins, may translocate into the nucleus to activate defense responses. For example, the barley MLA10 CC-NB-LRR (Coiled-coil-nucleotide-binding-site-leucine-rich repeat) R protein associates with the two repressors of PAMP-triggered immunity, hvWRKY1 and hvWRKY2, inside the nucleus in response to the fungal avirulence A10 effector, thereby de-repressing

PAMP mediated responses and initiating ETI (11). Another important example is the *Arabidopsis* Toll Interleukin-like Receptor (TIR) type of NB-LRR R protein, RRS1. RRS1 is an atypical R protein in that it has an additional WRKY domain characteristic of some plant transcription factors. RRS1 co-localizes with the corresponding *Ralstonia solanacearum* Avr protein, PopP2 and the nuclear localization of RRS1 is dependent on the PopP2 type III effector (12). Direct gene targets for these apparent transcriptional regulators are, however, unknown.

Ubiquitin-mediated post translational regulation is involved in many cellular processes, including hormone and stress responses (13). E3 ubiquitin ligases are key enzymes in the ubiquitination pathway as they directly catalyze the conjugation of ubiquitin to protein targets marking them for degradation by the 26S proteasome or activating them to assume their function in other biological processes (13). Many E3 ubiquitin ligases are implicated in defense responses. The SGT1 protein, for instance, associates with components of the SCF type E3 ubiquitin ligase complex and is required by many *R* genes for resistance (14, 15). Other E3 ubiquitin ligases such as the F-box protein COI1, the U-box protein SPL11 or the RING finger proteins RIN2 and RIN3, which interact with *Arabidopsis* RPM1 R protein in yeast, have also been implicated in plant defense responses (16-18).

If we are to understand how R protein activation is translated into signal transduction and primary gene expression, it is critical to identify the host proteins they interact with to mediate these responses. Protein complex purification schemes, yeast-two-hybrid screens, and *in-vivo* subcellular localization assays such as bimolecular fluorescence complementation should help in that direction. The identification of downstream targets for these signaling components, including DNA cis-elements responsive to transcription factors, will provide a platform for identifying the terminal stages of signal transduction pathways that translate signal perception into early gene expression and defense execution.

The maize *Rxo1* gene encodes a typical NB-LRR protein and confers resistance specifically to the bacterial leaf streak pathogen *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*) expressing the *avr* gene *avrRxo1* when expressed as a transgene in rice. Delivery of the *Xoc* Avr protein by the bacterial blight pathogen *Xoo* also elicits a strong HR in *Rxo1* expressing maize and rice plants (19).

In our previous microarray hybridizations in maize, we showed the upregulation of *ZmPti1b* encoding a protein kinase in response to *Xoo* expressing *avrRxo1*. To identify genes

encoding proteins that directly interact with RXO1, we used the entire coding sequence of this *R* gene as bait to screen a rice cDNA library. We report the isolation of several cDNA clones and the functional analysis of *Os1PVOZ* (*Os1 Plant Vascular One Zinc finger*, a putative transcription factor) and *OsATL6* (a putative E3 ubiquitin ligase) along with a rice homolog of *ZmPti1b*, *OsPti1a*, because of their potential roles in early signaling cascades that mediate *Rxo1* governed resistance. Using gene silencing, we show that while *OsPti1a* and *Os1PVOZ* are required for *Rxo1*-specified resistance, *OsATL6* is not required. Scanning of the rice genome for putative DNA binding sites suggests that Os1PVOZ regulates the expression of hundreds of genes with most coding for unknown proteins. Among the known proteins, approximately 40% encode signaling molecules including NB-LRR proteins, RLKs, and proteins involved in the generation of active oxygen species (AOS) and secondary metabolites. To our knowledge, this interaction between a transcription factor and an NB-LRR protein is the first of its kind to be investigated and shown to be required for effector-triggered immunity.

Results

ZmPti1b is differentially regulated in response to X. oryzae pv. oryzae (avrRxo1)

Microarray analyses were carried out to identify differentially regulated maize genes in response to infiltration with *Xoo* expressing *avrRxo1* as compared to the isogenic *Xoo* without *avrRxo1*. These analyses revealed a 3.6 fold upregulation of *ZmPti1b* 8 h after challenge with *Xoo (avrRxo1)*. We performed time course quantitative real-time PCR experiments using *ZmPti1b* specific primers to confirm this induction. *ZmPti1b* was induced as early as 8 hpi in response to *Xoo (avrRxo1)* with a six-fold change (Fig.14). This corroborates results observed with the microarray hybridizations and shows that *ZmPti1b* is differentially regulated between effector-triggered immunity and PAMP-triggered immunity.

RXO1 interacts with proteins that cluster into different functional groups

To identify proteins that directly interact with RXO1, we used the yeast-two-hybrid system. Screening of a rice cDNA library using the entire *Rxo1* coding sequence as bait identified seven cDNA clones, in frame with the GAL4 activation domain, from 4 x 10^6 transformants. These clones are in addition to four others that were previously isolated (Zhao et al., unpublished). Based on their nucleotide sequences, the 11 encoded proteins belong to 11

distinct classes (Table 4). Function of 5 genes encoding RXO1 interacting proteins were investigated using RNAi (B116, 907, 221, 232, and 409, see Table 4). RNAi lines regenerated from the rice transformation process for only two, B116 (*Os1PVOZ*), encoding a putative transcription factor, and 907 (*OsATL6*), a putative E3 ubiquitin ligase. No lines were regenerated for 221 encoding Flavon-3-hydroxylase, 232, encoding a 60KDa chaperone, and 409, encoding an ABC transporter. It is possible that the down regulation of these genes is lethal. *Os1PVOZ* and *OsATL6* may respectively be involved in the transcriptional and post-translational regulation of gene expression during defense, they were further characterized.

RNAi-mediated gene silencing of OsPti1a, Os1PVOZ, and OsATL6

The possible involvement of *OsPti1a*, *Os1PVOZ*, and *OsATL6* in *Rxo1* mediated resistance was investigated by using RNAi to down regulate the expression of these genes in rice plants expressing *Rxo1*. A fragment from each of the three genes was selected as RNAi trigger sequence and introduced into the pANDAβ destination vector (20) to create a hairpin overexpression cassette (Fig. 15A and 15B) to stably express in rice. More than twenty independent transgenic lines were generated for each construct and tested for correct expression of the transgene by RT-PCR using primers lying within the GUS-linker sequence (Fig. 15B and 15C: Only representative lines are shown). Expression of the hairpin cassette was also determined by quantitative real-time PCR (qRT-PCR) using primers lying within the trigger sequence (Fig. 15A). Plants expressing the hairpin constructs as indicated by the presence of the GUS linker also showed at least 2 fold upregulation of the trigger sequences as compared to those which show little or no GUS expression (Fig. 15C, and Fig. 16). To test for silencing of the endogenous gene, specific primers absent from the trigger sequences (Fig. 15A) were used to perform qRT-PCR. All plants expressing the silencing constructs showed clear down regulation of *OsPti1a*, *Os1PVOZ*, and *OsATL6* (Fig. 16).

Suppression of OsPti1a or Os1PVOZ, but not OsATL6, expression by RNAi abrogates the Rxo1 specified hypersensitive response

The effect of silencing of the three genes on the *Rxo1*-mediated HR was next investigated by challenging rice plants with *Xoo* expressing the *Xoc* type III effector *avrRxo1* [PXO99A (avrRxo1)]. Two days post inoculation (dpi), more than ten T0-*Ospti1a* and more than 20 T1-

Os1pvoz silenced lines showed clear water soaking (susceptibility) and complete loss of the HR (Fig. 17). These responses were identical to those observed in interactions of *Xoo* [PXO99A (p*avrRxo1*)] and kitaake rice plants without *Rxo1* or a regenerated line that did not express the silencing cassette. *Ospti1a* silenced lines exhibited a spontaneous lesion mimic cell death phenotype (Fig. 18), were severely impaired in seed production, showed a dwarf phenotype and tillered poorly.

In contrast to *Os1pvoz* and *Ospti1a,* more than 20 *Osatl6* silenced lines showed a strong HR, similar to regenerated lines that did not express the silencing cassette, and *Rxo1* control lines (Fig. 18). These data indicate that *Os1PVOZ* and *OsPti1a* are required for *Rxo1*-mediated resistance whereas *OsATL6* is dispensable.

Bioinformatic analysis suggests OsPVOZ transcription factors are master regulators of signal transduction

AtPVOZ transcription factors in *Arabidopsis* were demonstrated to bind a 38 bp DNA fragment containing the GCGTNx7ACGC palindromic sequence (GCGTN7 palindrome; 21). AtPVOZ2 binds to this palindromic sequence as a dimer. Two PVOZ transcription factors are found in the *Arabidopsis* genome and they share 62% sequence similarity in their predicted amino acid sequence. 142 promoters of the all Arabidopsis predicted genes bear the GCGTN7 palindrome (21).

We identified two *PVOZ* like transcription factors in rice, designated *Os1PVOZ* and *Os5PVOZ,* with 76% similarity to one another at the amino acid level. Os1PVOZ is 69% and 71% similar to the *Arabidopsis* AtPVOZ1 and AtPVOZ2, respectively, whereas Os5PVOZ shares 70% similarity with AtVOZ1 and 72% similarity with AtVOZ2. The rice genome was searched for the GCGTN7 palindrome within sequences one Kb upstream of the predicted translational start sites of predicted genes (Fig. 19 and Table 5). We found 328 promoters carrying the GCGTN7 palindrome, suggesting that OsPVOZ transcription regulate the expression of hundreds of genes.

Discussion

The *Rxo1*-mediated resistance response shares features of both effector-triggered immunity and PAMP mediated immunity.

Our first strategy was to use microarray analysis which would allow us to identify maize genes that are differentially regulated in the presence of *avrRxo1* as compared to its absence. We saw upregulation of several genes encoding signaling molecules (proteins involved in lipid and calcium signaling, in phosphorylation and transcriptional cascades) that may play roles in the regulation of defense responses.

Of 767 genes upregulated genes in response to *Xoo* [PXO99A (pavrRxo1)], we focus here on a tomato ortholog of *Pti1*, *ZmPti1b* that was upregulated during ETI as compared to PAMP Triggered Imunity in *Rxo1*-maize. Using qRT-PCR, we confirmed this upregulation in maize. Using gene silencing, we demonstrated that the rice ortholog, *OsPti1a*, is required for *Rxo1*-specified resistance in rice. Our data are consistent with findings in *Nicotiana bentamiana,* although no knockout or knockdown mutants have been studied in dicots. Overexpression of tomato *Pti1* in *N. benthamiana* accelerated *Prf/Pto* mediated HR in response to *P. syringae* pv. *tabaci* expressing *avrPto* suggesting *Pti1* is a positive regulator of the HR (22). Conversely, overexpression of *OsPti1a* in rice containing the blast disease resistance gene *Pish* impaired resistance against *M. oryzae* carrying the corresponding *avrPish*, as indicated by increased lesion on the leaves as compared to the HR. These plants were also more susceptible to a compatible strain of *Xoo*. Moreover, a null mutant for *OsPti1a* displayed resistance against a compatible race of *M. oryzae* (23). Incompatible interactions with *Xoo* were not tested, as no known bacterial blight *R* genes are found in the host plant Nipponbare. The null mutant rice plants exhibited a lesion mimic phenotype similar in appearance to the lesion mimic mutations that formed on our *OsPti1a* silenced lines. The impact of *Pti1* overexpression or knockout in rice with the *Pish* gene and interactions with compatible and incompatible *M. oryzae* suggest that *OsPti1a* acts as a negative regulator of ETI and as a positive regulator of disease susceptibility in rice. This apparent discrepancy with our results, where silencing of *OsPtia* obliterated ETI based resistance to *Xoo,* may be explained in part by the fact that *M. oryzae* is a hemibiotrophic pathogen and that the spontaneous lesions on *OsPti1a* mutant plants impair the ability of the fungus to initiate infection. *Ospti1a* associated phenotypes are dependent on *RAR1* (23), an important component of ETI that mediates resistance controlled by several NB-LRR proteins (24, 25). OsPti1a acting upstream of RAR1 may place this protein in a position to interact with NB-LRR proteins and possibly RXO1, but this has not been tested. Activation of RXO1 by AvrRxo1 could lead to OsPti1a phosphorylation of downstream targets, including transcription

factors that could translocate to the nucleus and activate primary gene expression that mediate *Rxo1*-governed innate immunity. Early induction of *ZmPti1b* may be a way for plant cells to amplify signal leading to the hypersensitive response.

Our second strategy was to identify *Rxo1*- interactors using the yeast-two-hybrid system. We focus here on two RXO1 interacting proteins, Os1PVOZ and OsATL6. RNAi-mediated suppression of *Os1PVOZ* obliterated *Rxo1*-mediated resistance. PVOZ transcription factors have been implicated in development (34) but never in defense. Computational analysis suggests that Os1PVOZ is involved in signaling cascades that promote signal amplification because of the many signaling proteins that are potential downstream targets for this transcription factor. Some of the potential targets include NB-LRR proteins and these may contribute to *Rxo1*-mediated resistance. An example has been described in *Arabidopsis* where TAO1, a TIR-NB-LRR protein acts additively with the CC-NB-LRR protein RPM1 to bring about specific full disease resistance to *P. syringae* expressing *avrB* but not *avrRpm1* (26). The atypical domain architecture of the TIR-NB-LRR R protein RRS1, which features an additional WRKY domain (12), suggests that some R proteins may recruit transcription factors upon activation to mediate their responses. To our knowledge however, no transcription factor has been shown to interact with an NB-LRR R protein and to be required for ETI. It is conceivable that upon RXO1 activation following delivery of AvrRxo1 inside plant cells Os1PVOZ is activated, possibly via postranslational modification, and then translocated into the nucleus where it regulates the expression of downstream gene targets. 132 of the predicted genes containing the palindrome either code for proteins of unknown function or reside upstream of sequences with transposons or retroelement homology. Of the remaining 196 predicted genes, more than 40% encode predicted signaling molecules including three NB-LRR proteins. One of these NB-LRR proteins has 53% similarity to RXO1 and a second one 58% similarity to Xa1, a protein conferring resistance to race 1 of *Xoo* (5). Other interesting genes include an ortholog of the sugar beet *Hs1pro-1* gene conferring gene for gene resistance to the beet cyst nematode (27) and an ortholog of *AtEXS-RLK* that has been implicated in developmental control in *Arabidopsis* (28). A *MAP kinase kinase kinase* (*MPKKK*) *OsMPKKK1*, several *Calcium dependent protein kinases* (*CDPKs*), as well as genes encoding protein phosphatases and proteins involved in the ubiquitination pathway are also found among the putative targets. GCGTN7 cis elements are also found in at least 16 predicted transcription factor encoding genes including those belonging to

MYB, *bZIP* and *NAC* families. Noticeable genes functioning in defense are those encoding peroxidase1, NADPH oxidoreductase, PR5 as well as several genes involved in the biosynthesis of secondary metabolites.

Downregulation of *OsATL6* did not reveal any obvious phenotype, we cannot however rule out a role in defense as we have not yet performed bacterial proliferation assays to assess its possible role in PTI and/or ETI. It is also possible that there is functional redundancy as *OsATL6* belongs to a gene family known as the *ATL* family in *Arabidopsis*. Fifteen *ATL* genes have been described in *Arabidopsis*. Two of these, *ATL2* and a homology of *OsATL6, ATL6*, are rapidly induced in response to chitin in seedlings (29). Using quantitative phosphoproteomics, another study showed a rapid increase in the phosphorylation status of ATL6 minutes after flagellin 22 treatment of *Arabidopsis* cells (30). Another rice family member, *EL5*, was also shown to be rapidly induced in response to elicitor treatment (31). In light of transcriptional and posttranslational regulation of *OsATL6* orthologs and family members in response to elicitor of defense responses, *OsATL6* may be involved in plant innate immunity by an as yet undetermined mechanism.

Our data identified key signaling components that mediate *Rxo1*-specified resistance and possibly resistance mediated by other *R* genes and provide a platform for dissecting ETI and/or PTI (Fig. 20). RNAi lines generated in this work provide valuable tools for use with targeted or global approaches to gain more insight into plant innate immunity, plant development and stress signaling to a greater extent.

Material and methods

Bait construction and yeast transformation

The full *Rxo1* coding sequence was PCR amplified as an *Eco*RI/S*al*I fragment using EcoRIRxo1f (**GAATTCCCCGGGATGGCAGAGATTGCTGTTCTT**), and SalIRxo1r primers (**GTCGACCATTTCCTTTTGAAAGCTGCT**) and cloned into the pBD-GAL4-Cam vector from the yeast-two-hybrid hybriZAP Kit (Stratagene Inc., La Jolla,) digested with the same enzymes. The resulting construct was transformed into yeast as previously described (33).

Rice cDNA yeast-two-hybrid library

The rice yeast-two-hybrid cDNA library used in this study was a kind gift from Dr. Frank White's lab (Department of plant pathology, Kansas State University). This library was generated using cDNA from the rice line IRBB7 carrying the *Xoo* resistance gene *Xa7*. cDNA samples were synthesized using RNA harvested from leaves 24 hours after challenge with an *Xoo* strain expressing the *avrXa7* type III effector gene. cDNA synthesis and yeast-two-hybrid cDNA library construction were performed using a Stratagene® Hybri ZAP2.1 cDNA library construction kit (Stratagene Inc., La Jolla). The average size of cDNA inserts is approximately 1.0 Kb (Yang, B. and White, F., unpublished data).

Screening of the transformed yeast clones

Co-transformed yeast strains with both bait and cDNA library clones were plated on selective media lacking the amino acids histidine, tryptophan, and leucine. Proliferating colonies were picked seven days thereafter and screened for β-galactosidase activity using a filter lift-up assay method (Stratagene Inc., La Jolla).Plasmids encoding putative interacting proteins were rescued into *E. co*li strain DH5α and sequenced using a GAL4-ADF primer (AGGGATGTTTAATACCACTAC). Sequence homology searches were then performed through GenBank (ncbi.nlm.nih.gov/BLAST/) and through the Rice Genome Annotation Project (rice.plantbiology.msu.edu).Rescued plasmids were co-transformed into yeast along with the *Rxo1* bait to repeat the experiment. It is possible that some of these may be false positives. More confirmatory studies are needed.

Quantitative RT-PCR and RT-PCR analyses

The same maize RNA samples from the microarray analyses with *Xoo* inoculations (see chapter 2 material and methods) were used for *ZmPtib* expression analysis. Rice 18S gene was used as an internal control. Three biological replications were performed and the fold changes were determined using the ΔΔCt method. *ZmPti1b* forward and reverse primers (CCGAACATGAGCATTGTTGT and GGAGAGAAGCAAACGCAAAG), and *Os18S* forward and reverse primers (ATGATAACTCGACCGATCGC and CTTGGATGTGGTAGCCGTTT) were used for the PCR reactions. To characterize the silenced lines, RNA was extracted from untreated leaves of transgenic and non transgenic rice plants using trizol (Invitrogen, Carlsbad). All RNA samples were subjected to DNAse I (Promega, Madison) treatment and 10ug of total

RNA was reverse transcribed using superscript III reverse transcriptase (Invitrogen, Carlsbad) according to manufacturer's protocols. cDNA derived from 100 ng of total RNA was used for qRT-PCR with the iCycler iQ (Bio-Rad, Hercules) using SYBR green Supermix in the PCR reactions (VWR, West Chester). RT-PCR were carried out using GUSF2 (ACCTCGCATTACCCTTACCGTGAA) and GUSR2 (ACGCGGTGATACATATCCAGCCAT) primers lying within the GUS-linker sequence to test for the expression of the RNAi-silencing transgene cassettes (Fig. 15B). The expression of the silencing cassettes was also tested using qRT-PCR with primers lying within the RNAi trigger sequence (Fig. 15B). Primers siPti1a forward and reverse (ATGGGCACAACGTGTGAAGATAGC, and TTGCTGGACTTGATGTCCCTGTGT), si-116 forward and reverse (CTCTGTTTGCTGCCCTCAGTTCAA and TGGCTTGTCAAAGAACAGCCACTC), and siOsL6 forward and reverse (AGGAGACCGAGGAAGAGAGGATCATA and TCGAATGCGAGCGCGGGAAA) were used to test for the transgene expression. Primers ePti1a forward and reverse (ACCGGAAGGAAACCTGTTGACCAT and ATACTGAACGCATAACGCCGCAAC), e-116 forward and reverse (ATCCAGCAGCAAATGGTTAGGCTG and TCCGGTTGTGACACTGCCTGATAA), and eOsL6 forward and reverse (TCTTCTTCCTCGGCTTCTTCTCCA and TTGACGGACTTGTGCGCCTTCA) were used to test for down regulation of the endogenous gene (Fig. 15A and Fig. 16). Rice 18S primers were used for internal control and the ΔΔCt method was applied to calculate fold changes.

Construction of gene silencing vectors and rice transformation

Fragments of 387bp, 334bp, 446bp, 391, 388, and 344 long for OsPti1a, Os1PVOZ, and OsATL6, 221, 232 and B409, respectively ,were PCR amplified from kitaake-rice leaf cDNA using Pfx DNA polymerase (Invitrogen, Carlsbad), cloned into pENTR/D-TOPO (Invitrogen, Carlsbad) and sequenced. Primers used to amplify each of these fragments are listed in Table 6 and are named siR-Pti1aF/R, siR-116F/R for Os1PVOZ, siR-OsL6F/R for OsATL6, siR-221F/R, siR-232F/R, and siR-B409F/R. All fragments were then excised with MluI and subjected to the LR recombinase reaction with pANDAβ (40) according to manufacturer's protocol (Invitrogen, Carlsbad). Recombinant vectors were electroporated into *Agrobacterium tumefaciens* strain EHA105.

Stable transgenic lines were developed from calli derived from immature embryos using *Agrobacterium*-mediated transformation essentially as previously described (32).

Bacterial culture and inoculation

Xoo strain *PXO99A* (*avrRxo1*) was cultured on TSA medium containing 100 ug/ml spectinomycin and 100ug/ml streptomycin for four days. Bacteria were then resuspended in ice cold water to a density of $5x10^8$ CFU/ml and infiltrated into fully expanded leaves of seeding plants for T0 plants and four week old plants for T1 plants

Computational analysis

PVOZ binding sites were queried against 1Kb upstream sequences of all rice gene models from the Rice Genome Annotation Project (rice.plantbiology.msu.edu) using a custom perl script. Cis element sequences were found using a custom, pattern-finding perl script which identifies exact sequence strings/motifs in a multifasta file considering both plus and minus strands. The output tables for each sequence motif gives locus ID, position in fasta, plus or minus strands in identified sequence.

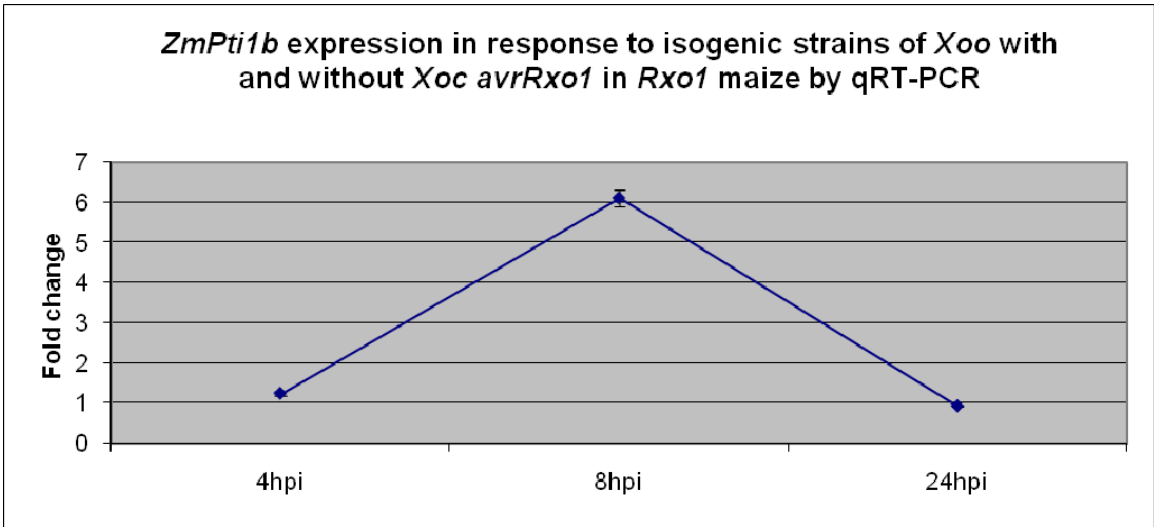

Figure 14 Time course analysis of *Zmpti1b* expression in *Rxo1*-maize by quantitative real-time PCR

RNA samples used for the microarray experiments (Chapter 2) were used to confirm the upregulation of *ZmPti1b* in response to *Xoo* (p*avrRxo1*) relative to the isogenic strain of *Xoo*. qRT-PCR was carried out using gene specific primers and rice 18S primers for loading controls. The ΔΔCt method was then used to calculate the average fold change values on three biological replications for each time point.

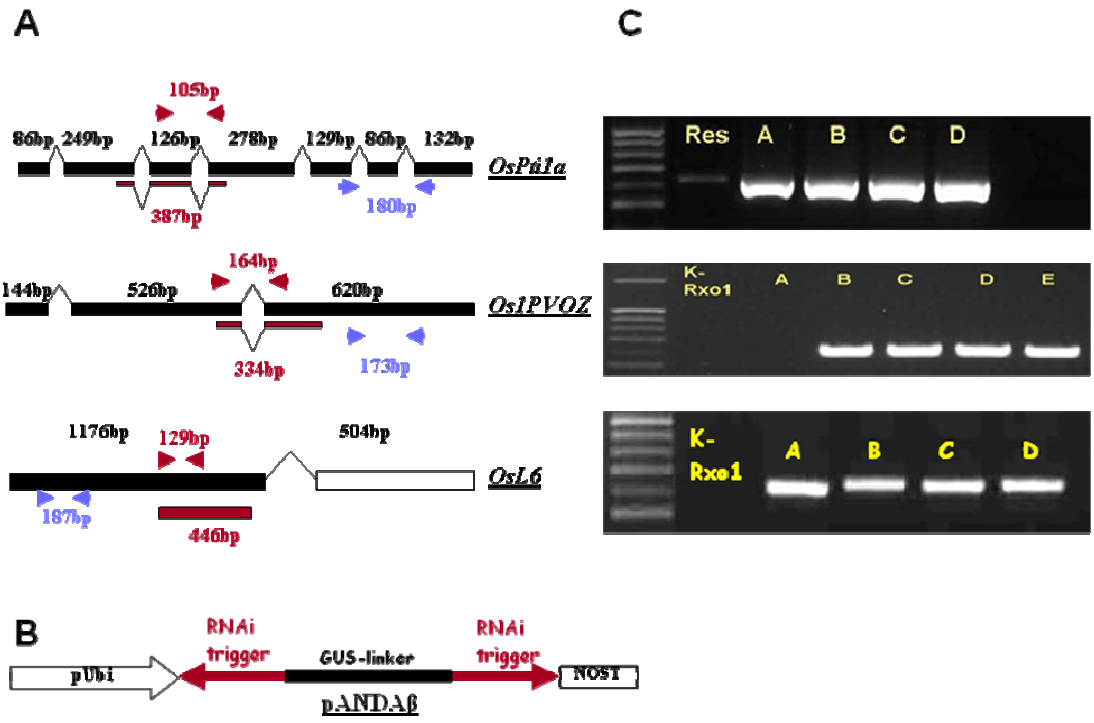

Figure 15 Gene silencing of *OsPti1a*, *Os1PVOZ*, and *OsATL6*.

(A) Structure of the three genes: Intron-Exon organizations with size of each exon indicated on top. The unfilled bar in *OsATL6* is only predicted, with no EST support. The maroon bars indicate the fragments used as RNAi trigger sequences with their respective size and map. Maroon arrows represent primers used in Fig. 3 (qRT-PCR) to test for expression of the silencing cassette and blue arrows represent primers used in qRT-PCR to test for silencing of the endogenous genes. **(B)** Diagram of the silencing vector, pANDAβ, indicating the inverted repetition of the trigger flanking the GUS-linker sequence after successful recombination. Expression is driven by the strong maize ubiquitin promoter (pUbi) and NOST is the NOS terminator. **(C)** RT-PCR showing the expression of the GUS-linker. "Res" is a line that has gone through the transformation process but still shows expression of *Rxo1*-mediated resistance. K-*Rxo1* is the rice parental untransformed line expressing *Rxo1*. A, B, C, D, and E lines are independent transgenic lines. Top gel shows expression of the GUS fragment in *OsPti1a* T0 lines, middle, *Os1PVOZ* T1 lines, and bottom, *OsATL6* T0 lines. All primer sequences are shown in Table 6.

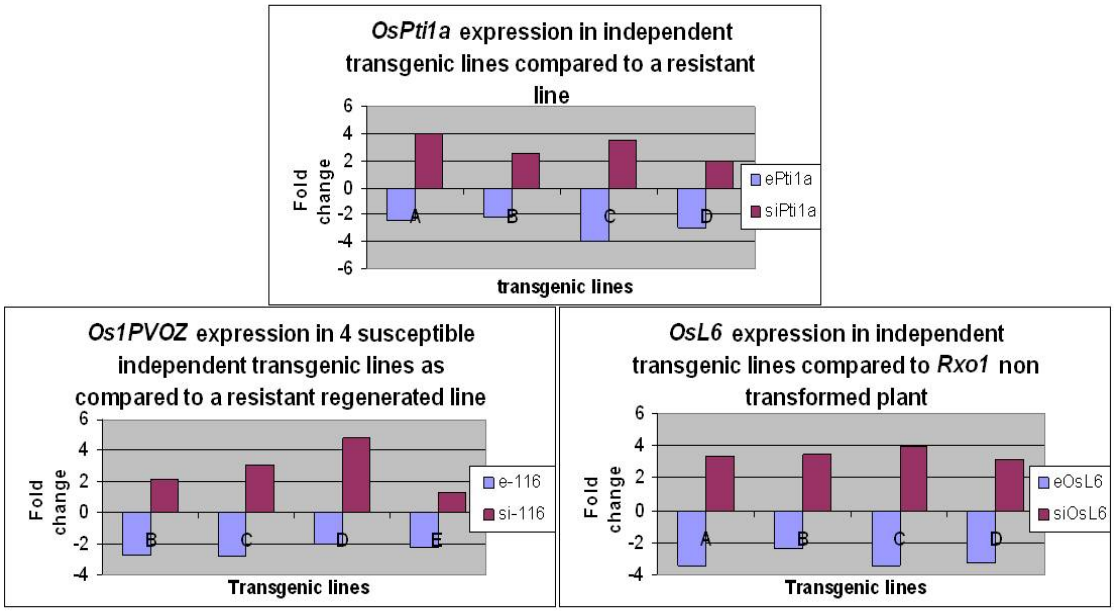

Figure 16 Expression of the silencing cassette and the endogenous genes.

ePti1a is the expression of the **e**ndogenous gene as compared to a resistant regenerated line and siPti1a represents the expression of the RNAi trigger sequence. The same is applicable to *Os1PVOZ* and *OsATL6*. A, B, C, D, and E are independent transgenic lines showing upregulation of the silencing cassette and downregulation of the corresponding endogenous gene when compared to a resistant regenerated line or to *Rxo1* line in the case of *OsATL6*

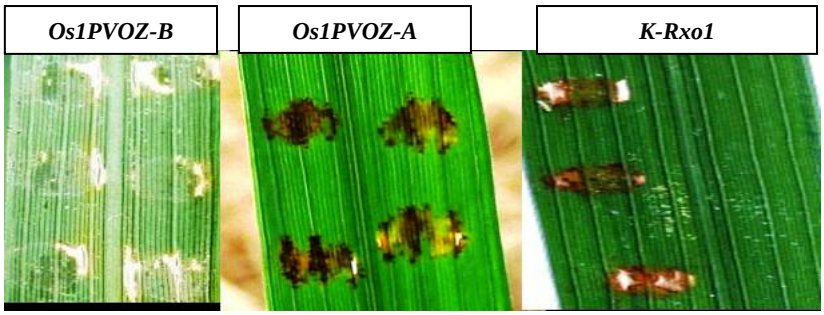

Figure 17 Phenotypic analysis of *Os1pvoz* silenced lines

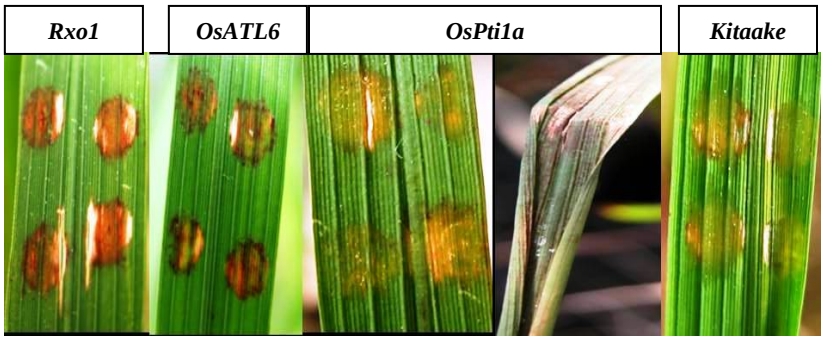

Figure 18 Phenotypic analysis of *Os1pti1a and Osatl6* silenced lines

Rxo1-rice plants were stably transformed with an RNAi silencing cassette. Plants were challenged with *Xoo* (*avrRxo1*).

17. Two days post inoculation (dpi) *Os1PVOZ-B* shows water soaking typical of disease susceptibility while *Os1PVOZ-A* that did not show GUS expression as well as the Kitaake-*Rxo1* plant show strong hypersensitive response, a hallmark of resistance.

18. *OsPti1a* silenced lines *and kitaake* (does not express *Rxo1*) show water soaking (*OsPti1a* left leaf, 2 dpi) and leaf curling (right leaf, 7dpi) typical of disease susceptibility while *Kitaake-Rxo1* and *OsATL6* silenced line exhibit strong hypersensitive response 2 days after challenge with *Xoo* (*avrRxo1*)

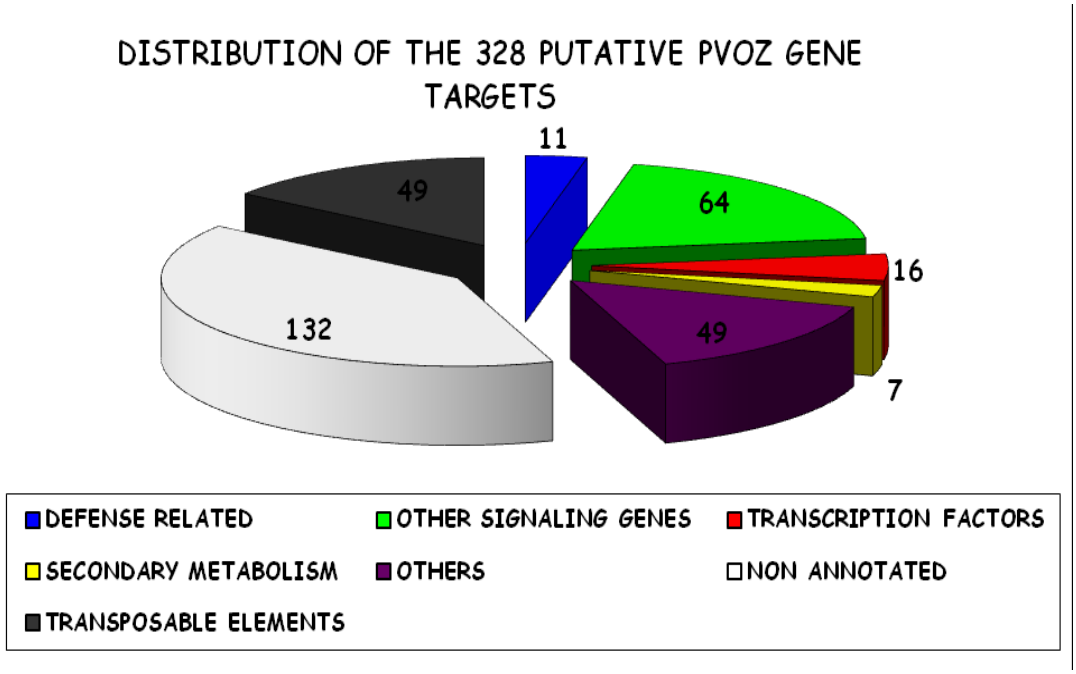

Figure 19 Functional distribution of OsPVOZ putative gene targets.

The Pie chart shows the clustering into seven categories of all rice gene models from the Rice Genome Annotation Project database containing the GCGTNx7ACGC palindrome sequence within 1KB upstream of their ATG start codon . These may be putative targets for OsPVOZ transcription factors

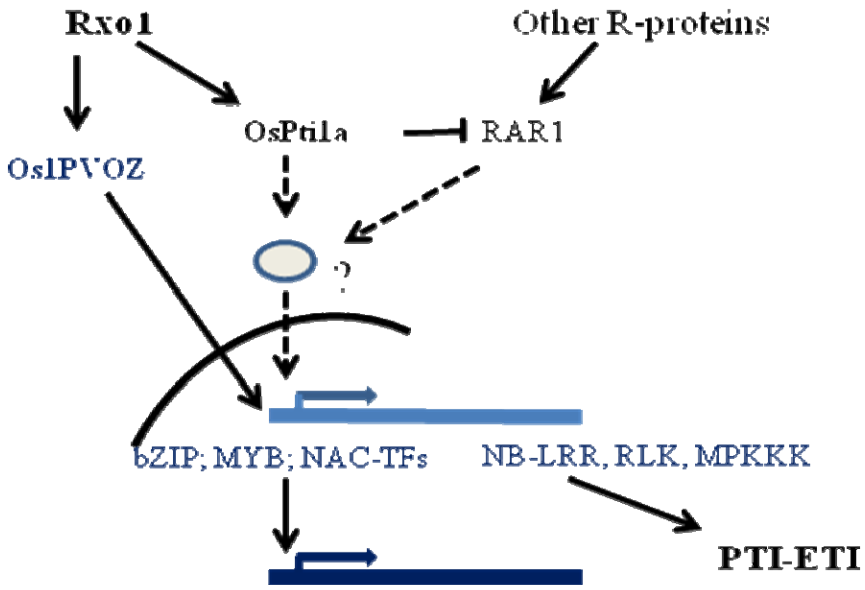

Figure 20 Model for *Rxo1*-mediated resistance

OsPti1a may phosphorylate and activate other signaling molecules including TFs that may translocate into the nucleus. OsPti1 may also inhibit RAR1 dependent responses. OsPVOZ may activate a cascade of signaling via massive transcriptional activation of signaling genes

Table 4 cDNA clones isolated using a yeast-two-hybrid screen with *Rxo1* as the bait

Designation	Predicted protein	E-value
B116	Rice putative vascular plant one zinc finger protein: Os1PVOZ	3e-165
907	Rice RING-H2 zinc finger protein ATATL6-like: OsATL6	3e-24
232	60KDa chaperonin alpha subunit	2e-134
818	Leaf senescence related protein-like	1e-87
230	Similar to tobacco 16kDa Mb Protein	4e-79
221	Flavon-3-hydroxylase like protein	8e-144
908	Putative phosphoadenylyl-sulfate reductase	8e-62
845	Putative aminolevulinate dehydratase	e-104
B79	Rice unknown protein Rubber elongation factor domain	3e-73
B121	Rice UDP-glucuronic acid decarboxylase	5e-147
B409	Rice MRP-like ABC transporter	5e-58

Table 5 Putative PVOZ gene targets bearing the GCGTNx7ACGC palindrome sequence within 1KB upstream of their ATG start codon

Locus ID	Putative Function
LOC_Os03g61270	1,4-beta-D-mannan endohydrolase precursor, putative, expressed
LOC_Os04g30760	3-oxoacyl-reductase, chloroplast precursor, putative, expressed
LOC_Os03g60370	acid phosphatase, putative, expressed
LOC_Os03g60370	acid phosphatase, putative, expressed
LOC_Os08g09200	aconitate hydratase, cytoplasmic, putative, expressed
LOC_Os02g48720	ADP,ATP carrier protein, mitochondrial precursor, putative, expressed
LOC_Os01g59790	ADP-ribosylation factor, putative, expressed
LOC_Os02g47110	ADP-ribosylation factor, putative, expressed
LOC_Os03g59740	ADP-ribosylation factor, putative, expressed
LOC_Os12g09320	amino acid carrier, putative
LOC_Os04g47780	amino acid transport protein, putative, expressed
LOC_Os08g23590	ankyrin-like protein, putative
LOC_Os08g23590	ankyrin-like protein, putative
LOC_Os01g53330	anthocyanidin 5,3-O-glucosyltransferase, putative, expressed
LOC_Os04g56290	apospory-associated protein C, putative, expressed
LOC_Os03g55330	arginine N-methyltransferase 2, putative, expressed
LOC_Os08g03510	ATBPM1, putative
LOC_Os01g64490	ATP binding protein, putative, expressed
LOC_Os04g56590	ATP/GTP binding protein, putative, expressed

LOC_Os09g39910	ATP-binding cassette sub-family F member 2, putative, expressed
LOC_Os01g66330	ATP-dependent Clp protease ATP-binding subunit clpX, putative, expressed
LOC_Os03g31490	ATP-dependent protease Clp, ATPase subunit, putative, expressed
LOC_Os06g09660	auxin response factor 19, putative, expressed
LOC_Os06g35900	brassinazole-resistant 1 protein, putative, expressed
LOC_Os05g41270	calcium-dependent protein kinase, isoform 2, putative, expressed
LOC_Os01g66240	catalytic/ hydrolase, putative, expressed
LOC_Os02g46080	catalytic/ protein phosphatase type 2C, putative, expressed
LOC_Os10g06580	cell division control protein 2 homolog 3, putative, expressed
LOC_Os03g01810	charged multivesicular body protein 3, putative, expressed
LOC_Os03g16000	clathrin binding protein, putative, expressed
LOC_Os07g48910	collagen-like protein 2, putative, expressed
LOC_Os01g66230	csAtPR5, putative, expressed
LOC_Os03g45210	cupin, RmlC-type, putative, expressed
LOC_Os03g45250	cupin, RmlC-type, putative, expressed
LOC_Os05g29750	cytochrome P450 71E1, putative, expressed
LOC_Os04g02860	disease resistance protein RPM1, putative RXO1
LOC_Os11g29030	disease resistance protein RPM1, putative
LOC_Os04g50090	DNA binding protein, putative bZIP
LOC_Os01g14430	DNA binding protein, putative, expressed
LOC_Os02g36890	DNA binding protein, putative, expressed MYB
LOC_Os07g22400	DNA primase large subunit, putative, expressed
LOC_Os03g06120	domain of unknown function DUF614 containing protein, expressed Ca2+ transport
LOC_Os07g42730	EF hand family protein, expressed
LOC_Os09g30490	EF hand family protein, expressed
LOC_Os07g46370	EMB2221, putative, expressed
LOC_Os07g46370	EMB2221, putative, expressed
LOC_Os04g42140	eukaryotic initiation factor iso-4F subunit p82-34, putative, expressed
LOC_Os02g25870	eukaryotic peptide chain release factor GTP-binding subunit, putative, expressed
LOC_Os07g40580	eukaryotic translation initiation factor 5A, putative, expressed
LOC_Os03g06700	fatty acid elongase, putative, expressed
LOC_Os01g41430	flavonol-3-O-glycoside-7-O-glucosyltransferase 1, putative, expressed
LOC_Os03g58900	galactosyltransferase/ transferase, transferring hexosyl groups, putative, expressed
LOC_Os03g14730	gibberellin receptor GID1L2, putative, expressed
LOC_Os03g52460	glucose-1-phosphate adenylyltransferase large subunit 3, chloroplast precursor, putative, expressed
LOC_Os03g15840	glycosyl transferase, group 1 family protein, putative, expressed
LOC_Os08g44390	grancalcin, putative, expressed
LOC_Os11g04570	GRAS family transcription factor containing protein, expressed
LOC_Os01g51250	Grave disease carrier protein, putative, expressed
LOC_Os02g45570	growth-regulating factor, putative, expressed
LOC_Os02g44860	GSDL-motif lipase, putative, expressed
LOC_Os12g06620	*HEAT-like, putative, expressed*
LOC_Os09g31090	HECT-domain, putative, expressed
LOC_Os09g29460	homeobox-leucine zipper protein ATHB-6, putative,

LOC_Os02g14540	hydroquinone glucosyltransferase, putative
LOC_Os11g38650	hydroquinone glucosyltransferase, putative, expressed
LOC_Os06g17250	indole-3-acetate beta-glucosyltransferase, putative
LOC_Os04g42920	isocitrate dehydrogenase, chloroplast precursor, putative, expressed
LOC_Os12g09720	jasmonate-induced protein, putative, expressed
LOC_Os10g32990	leucine-rich repeat receptor protein kinase EXS precursor, putative, expressed
LOC_Os04g53830	leucoanthocyanidin reductase, putative, expressed
LOC_Os11g04580	long cell-linked locus protein, putative
LOC_Os12g03790	lyase, putative, expressed
LOC_Os03g41070	metal transporter Nramp2, putative
LOC_Os07g15460	metal transporter Nramp6, putative, expressed
LOC_Os01g51530	methyltransferase, putative, expressed
LOC_Os01g73020	mitochondrial import inner membrane translocase subunit TIM16, putative, expressed
LOC_Os11g03710	mitochondrial import inner membrane translocase subunit Tim8, putative, expressed
LOC_Os01g73550	mitochondrial-processing peptidase alpha subunit, mitochondrial precursor, putative, expressed
LOC_Os01g50370	mitogen-activated protein kinase kinase kinase 1, putative, expressed
LOC_Os11g08210	NAC domain-containing protein 71, putative, expressed
LOC_Os03g31690	N-acetyltransferase/ amino-acid N-acetyltransferase, putative, expressed
LOC_Os04g37480	NAD(P)H-dependent oxidoreductase, putative, expressed
LOC_Os02g02680	NB-LRR disease resistance protein, putative, expressed similar to XA1
LOC_Os01g63690	nematode-resistance protein, putative, expressed HS1-pro
LOC_Os11g07916	NFU3, putative, expressed
LOC_Os03g39050	no apical meristem protein
LOC_Os03g39100	no apical meristem protein
LOC_Os05g25960	no apical meristem protein
LOC_Os07g27340	no apical meristem protein
LOC_Os07g27330	no apical meristem protein, expressed
LOC_Os08g42010	nodulin-like protein, putative, expressed
LOC_Os08g42000	nuclear transport factor 2, putative, expressed
LOC_Os02g45480	nucleic acid binding protein, putative, expressed
LOC_Os12g06870	*nucleoporin autopeptidase family protein, expressed*
LOC_Os12g06870	nucleoporin autopeptidase family protein, expressed
LOC_Os02g18650	pectinesterase-2 precursor, putative, expressed
LOC_Os06g41040	pentatricopeptide repeat protein PPR1106-17, putative, expressed
LOC_Os03g07234	pentatricopeptide repeat protein PPR986-12, putative
LOC_Os05g31160	peptide chain release factor 2, putative, expressed
LOC_Os07g01400	peroxidase 1 precursor, putative, expressed
LOC_Os03g49210	pescadillo, putative, expressed
LOC_Os02g05400	phosphatidate cytidylyltransferase, putative
LOC_Os08g01390	phosphatidylinositol-4-phosphate 5-Kinase family protein, expressed
LOC_Os04g46960	phospholipid hydroperoxide glutathione peroxidase, putative, expressed
LOC_Os04g28460	phospholipid-transporting ATPase 2, putative, expressed
LOC_Os12g21890	phosphoprotein phosphatase, putative, expressed
LOC_Os01g62990	pollen-specific kinase partner protein, putative, expressed
LOC_Os10g36760	prolyl carboxypeptidase like protein, putative, expressed

LOC_Os03g56930	protein app1, putative, expressed
LOC_Os10g34740	protein arginine N-methyltransferase 6, putative, expressed
LOC_Os08g03260	protein binding protein, putative RING
LOC_Os02g36740	protein binding protein, putative, expressed RING
LOC_Os05g39610	protein binding protein, putative, expressed LZ
LOC_Os11g43030	protein binding protein, putative, expressed RING
LOC_Os05g07900	protein brittle-1, chloroplast precursor, putative, expressed
LOC_Os02g54780	protein CYPRO4, putative, expressed
LOC_Os04g15910	protein dimerization, putative
LOC_Os05g41900	protein translation factor SUI1, putative, expressed
LOC_Os01g68370	protein viviparous, putative, expressed
LOC_Os01g56010	protein Z, putative, expressed
LOC_Os01g44394	rae1-like protein, putative, expressed
LOC_Os04g38610	RAN guanine nucleotide release factor, putative, expressed
LOC_Os04g38610	RAN guanine nucleotide release factor, putative, expressed
LOC_Os05g28290	ran-binding protein 1 homolog c, putative, expressed
LOC_Os03g17000	RHM1, putative, expressed
LOC_Os03g11220	RPGR, putative, expressed
LOC_Os03g41080	seed maturation protein PM23, putative, expressed
LOC_Os01g47262	STAM-binding protein, putative, expressed
LOC_Os04g14450	tetratricopeptide-like helical, putative, expressed
LOC_Os07g40750	tetratricopeptide-like helical, putative, expressed
LOC_Os07g08840	thioredoxin H-type, putative, expressed
LOC_Os03g17010	THO complex subunit 4, putative, expressed
LOC_Os02g47370	transcription factor GT-3b, putative, expressed
LOC_Os06g04580	transcription factor TFIIF, putative, expressed
LOC_Os01g03570	transcription factor X1, putative, expressed
LOC_Os01g52710	transferase, transferring glycosyl groups, putative, expressed
LOC_Os12g37510	transferase, transferring glycosyl groups, putative, expressed
LOC_Os07g08070	transmembrane BAX inhibitor motif-containing protein 4, putative, expressed
LOC_Os05g43850	tubby-related protein 1, putative, expressed
LOC_Os02g43760	ubiquitin carboxyl-terminal hydrolase isozyme L3, putative, expressed
LOC_Os04g57220	ubiquitin-conjugating enzyme E2-17 kDa, putative, expressed
LOC_Os04g58800	ubiquitin-conjugating enzyme spm2, putative, expressed
LOC_Os01g68940	ubiquitin-like protein SMT3, putative, expressed
LOC_Os01g68950	ubiquitin-like protein SMT3, putative, expressed
LOC_Os11g36490	U-box protein, putative
LOC_Os08g44510	UDP-N-acetylglucosamine--peptide N-acetylglucosaminyltransferase SPINDLY, putative, expressed
LOC_Os04g33300	uridylate kinase, putative, expressed
LOC_Os03g14690	vacuolar ATP synthase 91 kDa subunit, putative, expressed
LOC_Os01g37910	vacuolar processing enzyme precursor, putative, expressed
LOC_Os03g60560	ZFP16-2, putative, expressed light response
LOC_Os01g51710	zinc finger protein, putative, expressed
LOC_Os08g31720	zinc finger, C3HC4 type family protein
LOC_Os03g42820	zinc ion binding protein, putative, expressed
Locus ID	**Putative Function**
LOC_Os01g01500	conserved hypothetical protein

LOC_Os01g04560	conserved hypothetical protein
LOC_Os01g07000	conserved hypothetical protein
LOC_Os04g54490	conserved hypothetical protein
LOC_Os05g26026	conserved hypothetical protein
LOC_Os05g26049	conserved hypothetical protein
LOC_Os05g27749	conserved hypothetical protein
LOC_Os05g28129	conserved hypothetical protein
LOC_Os06g41620	conserved hypothetical protein
LOC_Os07g17520	conserved hypothetical protein
LOC_Os07g22760	conserved hypothetical protein
LOC_Os07g27280	conserved hypothetical protein
LOC_Os08g21650	conserved hypothetical protein
LOC_Os09g27410	conserved hypothetical protein
LOC_Os10g12650	conserved hypothetical protein
LOC_Os10g29040	conserved hypothetical protein
LOC_Os11g45410	conserved hypothetical protein
LOC_Os12g15490	conserved hypothetical protein
LOC_Os01g11820	expressed protein
LOC_Os01g13780	expressed protein
LOC_Os01g24350	expressed protein
LOC_Os01g29280	expressed protein
LOC_Os01g34050	expressed protein
LOC_Os01g38650	expressed protein
LOC_Os01g50890	expressed protein
LOC_Os01g57840	expressed protein
LOC_Os01g65610	expressed protein
LOC_Os01g66340	expressed protein
LOC_Os01g66480	expressed protein
LOC_Os02g14850	expressed protein
LOC_Os02g35540	expressed protein
LOC_Os02g51390	expressed protein
LOC_Os02g58110	expressed protein
LOC_Os03g19080	expressed protein
LOC_Os03g21040	expressed protein
LOC_Os03g43350	expressed protein
LOC_Os03g53740	expressed protein
LOC_Os03g58690	expressed protein
LOC_Os04g31140	expressed protein
LOC_Os04g35480	expressed protein
LOC_Os04g35490	expressed protein
LOC_Os04g35864	expressed protein
LOC_Os04g42940	expressed protein
LOC_Os04g46950	expressed protein
LOC_Os04g53380	expressed protein
LOC_Os04g56580	expressed protein
LOC_Os05g02250	expressed protein
LOC_Os05g02970	expressed protein
LOC_Os05g15350	expressed protein

LOC_Os05g23650	expressed protein
LOC_Os05g31890	expressed protein
LOC_Os06g45610	expressed protein
LOC_Os07g12100	expressed protein
LOC_Os07g12780	expressed protein
LOC_Os07g39830	expressed protein
LOC_Os07g42390	expressed protein
LOC_Os07g48490	expressed protein- among the 259 common genes
LOC_Os08g06940	expressed protein
LOC_Os08g07320	expressed protein
LOC_Os08g10410	expressed protein
LOC_Os08g34800	expressed protein
LOC_Os08g40230	expressed protein
LOC_Os08g43620	expressed protein
LOC_Os09g08580	expressed protein
LOC_Os09g13920	expressed protein
LOC_Os09g21470	expressed protein
LOC_Os09g33510	expressed protein
LOC_Os09g35540	expressed protein
LOC_Os09g37080	expressed protein
LOC_Os09g37344	expressed protein
LOC_Os10g07440	expressed protein
LOC_Os10g09684	expressed protein
LOC_Os10g11310	expressed protein
LOC_Os10g20510	expressed protein
LOC_Os10g35800	expressed protein
LOC_Os10g42196	expressed protein
LOC_Os11g03720	expressed protein
LOC_Os11g09940	expressed protein
LOC_Os11g37670	expressed protein
LOC_Os12g37519	expressed protein
LOC_Os01g50880	hypothetical protein
LOC_Os01g51000	hypothetical protein
LOC_Os01g57140	hypothetical protein
LOC_Os02g07000	hypothetical protein
LOC_Os02g07470	hypothetical protein
LOC_Os02g10410	hypothetical protein
LOC_Os02g28450	hypothetical protein
LOC_Os02g28530	hypothetical protein
LOC_Os02g45470	hypothetical protein
LOC_Os02g48710	hypothetical protein
LOC_Os02g53760	hypothetical protein
LOC_Os03g10550	hypothetical protein
LOC_Os03g39090	hypothetical protein
LOC_Os03g50710	hypothetical protein
LOC_Os03g58280	hypothetical protein
LOC_Os04g13260	hypothetical protein
LOC_Os04g15670	hypothetical protein

LOC_Os04g35710	hypothetical protein
LOC_Os04g41010	hypothetical protein
LOC_Os05g13430	hypothetical protein
LOC_Os05g13670	hypothetical protein
LOC_Os05g32020	hypothetical protein
LOC_Os06g03020	hypothetical protein
LOC_Os06g19420	hypothetical protein
LOC_Os06g35890	hypothetical protein
LOC_Os06g38150	hypothetical protein
LOC_Os06g39100	hypothetical protein
LOC_Os06g43010	hypothetical protein
LOC_Os06g43070	hypothetical protein
LOC_Os07g06240	hypothetical protein
LOC_Os07g12440	hypothetical protein
LOC_Os07g17490	hypothetical protein
LOC_Os07g27980	hypothetical protein
LOC_Os07g27980	hypothetical protein
LOC_Os07g38710	hypothetical protein
LOC_Os07g38880	hypothetical protein
LOC_Os08g03750	hypothetical protein
LOC_Os08g06260	hypothetical protein
LOC_Os08g07020	hypothetical protein
LOC_Os08g11270	hypothetical protein
LOC_Os08g26030	hypothetical protein
LOC_Os09g04910	hypothetical protein
LOC_Os09g08410	hypothetical protein
LOC_Os09g21590	hypothetical protein
LOC_Os09g24430	hypothetical protein
LOC_Os09g25630	hypothetical protein
LOC_Os09g25630	hypothetical protein
LOC_Os09g32880	hypothetical protein
LOC_Os10g33954	hypothetical protein
LOC_Os11g03080	hypothetical protein
LOC_Os11g08420	hypothetical protein
LOC_Os11g22360	hypothetical protein
LOC_Os11g40330	hypothetical protein
LOC_Os11g45140	hypothetical protein
LOC_Os12g08010	hypothetical protein
LOC_Os11g45420	retrotransposon protein, putative, LINE subclass
LOC_Os04g01220	retrotransposon protein, putative, Ty1-copia subclass
LOC_Os06g47070	retrotransposon protein, putative, Ty1-copia subclass
LOC_Os07g15890	retrotransposon protein, putative, Ty1-copia subclass
LOC_Os08g30360	retrotransposon protein, putative, Ty1-copia subclass
LOC_Os11g03940	retrotransposon protein, putative, Ty1-copia subclass
LOC_Os03g33140	retrotransposon protein, putative, Ty3-gypsy subclass
LOC_Os03g33328	retrotransposon protein, putative, Ty3-gypsy subclass
LOC_Os04g16870	retrotransposon protein, putative, Ty3-gypsy subclass
LOC_Os04g22590	retrotransposon protein, putative, Ty3-gypsy subclass

LOC_Os05g17370	retrotransposon protein, putative, Ty3-gypsy subclass
LOC_Os05g19350	retrotransposon protein, putative, Ty3-gypsy subclass
LOC_Os09g09240	retrotransposon protein, putative, Ty3-gypsy subclass
LOC_Os11g44640	retrotransposon protein, putative, Ty3-gypsy subclass
LOC_Os01g16570	retrotransposon protein, putative, unclassified
LOC_Os01g54680	retrotransposon protein, putative, unclassified
LOC_Os03g19660	retrotransposon protein, putative, unclassified
LOC_Os03g47410	retrotransposon protein, putative, unclassified
LOC_Os03g58270	retrotransposon protein, putative, unclassified
LOC_Os04g02300	retrotransposon protein, putative, unclassified
LOC_Os04g05450	retrotransposon protein, putative, unclassified
LOC_Os04g35460	retrotransposon protein, putative, unclassified
LOC_Os05g00998	retrotransposon protein, putative, unclassified
LOC_Os05g20790	retrotransposon protein, putative, unclassified
LOC_Os05g27060	retrotransposon protein, putative, unclassified
LOC_Os05g27060	retrotransposon protein, putative, unclassified
LOC_Os05g31210	retrotransposon protein, putative, unclassified
LOC_Os07g20210	retrotransposon protein, putative, unclassified
LOC_Os07g25560	retrotransposon protein, putative, unclassified
LOC_Os08g13970	retrotransposon protein, putative, unclassified
LOC_Os08g15660	retrotransposon protein, putative, unclassified
LOC_Os08g41020	retrotransposon protein, putative, unclassified
LOC_Os09g13150	retrotransposon protein, putative, unclassified
LOC_Os10g41610	retrotransposon protein, putative, unclassified
LOC_Os12g09260	retrotransposon protein, putative, unclassified
LOC_Os12g14880	retrotransposon protein, putative, unclassified
LOC_Os12g09210	retrotransposon protein, putative, unclassified, expressed
LOC_Os04g16850	retrotransposon, putative, centromere-specific
LOC_Os02g28370	transposon protein, putative, CACTA, En/Spm sub-class
LOC_Os02g34900	transposon protein, putative, CACTA, En/Spm sub-class
LOC_Os04g11410	transposon protein, putative, CACTA, En/Spm sub-class
LOC_Os08g34590	transposon protein, putative, CACTA, En/Spm sub-class
LOC_Os09g12050	transposon protein, putative, CACTA, En/Spm sub-class
LOC_Os11g42980	transposon protein, putative, CACTA, En/Spm sub-class
LOC_Os12g09330	transposon protein, putative, Mariner sub-class
LOC_Os01g53320	transposon protein, putative, Mutator sub-class
LOC_Os07g40760	transposon protein, putative, Mutator sub-class
LOC_Os07g41670	transposon protein, putative, unclassified
LOC_Os11g14270	transposon protein, putative, unclassified

Table 6 List of primers used in this work

Primer name	**Primer sequence**
Os18SF	**Atgataactcgaccgatcgc**
Os18SR	**Cttggatgtggtagccgttt**
Si-116F	**CTCTGTTTGCTGCCCTCAGTTCAA**
Si-116R	**TGGCTTGTCAAAGAACAGCCACTC**
siOsL6F	**AGGAGACCGAGGAAGAGAGGATCATA**

SiOsL6R	**TCGAATGCGAGCGCGGGAAA**
siPti1aF	**ATGGGCACAACGTGTGAAGATAGC**
siPti1aR	**TTGCTGGACTTGATGTCCCTGTGT**
e-116F	**ATCCAGCAGCAAATGGTTAGGCTG**
e-116R	**TCCGGTTGTGACACTGCCTGATAA**
ePti1aF	**ACCGGAAGGAAACCTGTTGACCAT**
ePti1aR	**ATACTGAACGCATAACGCCGCAAC**
ZmPti1bF	**CCGAACATGAGCATTGTTGT**
ZmPti1Br	**GGAGAGAAGCAAACGCAAAG**
GUSF2	**ACCTCGCATTACCCTTACCGTGAA**
GUSR2	**ACGCGGTGATACATATCCAGCCAT**
RXOCF	**TCACTTCGGATCATTGCTGTCTGG**
RXOCR	**CATGCACGGCAGTCAAAGTTGGAT**
EcoRIRxo1f	**GAATTCCCCGGGATGGCAGAGATTGCTGTTCTT**
SalIRxo1r	**GTCGACCATTTCCTTTTGAAAGCTGCT**
eOsL6F	**TCTTCTTCCTCGGCTTCTTCTCCA**
eOsL6R	**TTGACGGACTTGTGCGCCTTCA**
siR-116F	**CACCAGGCACAATGCCTGTGA**
siR-116R	**GACCTCTTCAGACCCCCAAAGTC**
siR-OsL6	**CACCaggagaccgaggaagagaggatcata**
siR-OsL6	**CCTCCGCGTCGACCTTGAT**
siR-Pti1aF	**caccTGGTGAAGGTTCCTTTGGCAGAGT**
siR-Pti1aR	**TTGCTGGACTTGATGTCCCTGTGT**
siR-221F	**CACCGCTTCTTCCAGGTGCTCAACCAT**
siR-221F	**TATGCTGCTCCTGCTCACCAAGAA**
siR-232F	**CACCAGCATAAAGGAGATTCTTCCA**
siR-232R	**AGTTTCGGAGAGTTCCTTCTTTAGC**
siR-B409F	**CACCAAATTTGGCAGGCCTTGGAA**
siR-B409R	**GGGTTGCGTACTCTTGAACC**
ZIMF	**TCGAATCAATTAACAGCCAAA**
ZIMR	**TCGGAGACGGAGGATTGTAG**
ABCF	**GTCAACAGCAGTGCACGA**
ABCR	**ATCCAGTAAAATTCCTTGTCG**
GST7F	**GGCTCATTACTGTTATTTCAGCAC**
GST7R	**TGGTTGCAGCAGTAGTTTGG**
PPCKF	**CAGCCTTCTTATGGCTGAGG**
PPCKR	**TTCATTCAAGGTTCCACAG**
CBPF	**ATGTTCTTTAGCTCGTCGGC**
CBPR	**ATTTCCGTCCCATGTCACAC**
GAL4-ADF	**AGGGATGTTTAATACCACTAC**
GAL4-ADR	**GCACAGTTGAAGTGAACTTGC**

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Conclusions and Perspectives

Our microarray data suggest that host and non host resistances display similar transcriptional outputs. The main difference lies within the kinetics in the expression of genes. This may be due to different biochemical signatures generated by different suites of effectors delivered into the plant cell by *Xoo* and *Ba*. Analysis of promoter elements of rice homologs of maize genes revealed the enrichment of novel putative cis elements within the promoter of early induced genes as well as the overrepresentation of the ABRE core. The early induction of defense signal pathway genes, such as the maize homolog of the Pto-kinase-interactor1 (*Pti1*), *ZmPti1b*, were observed. LePti1 interact with Pto, is phosphorylated by Pto and is involved in HR in tomato. Gene silencing revealed that rice a homolog of *ZmPti1b*, *Ospti1a*, was required for *Rxo1*-mediated resistance. RNAi-mediated gene silencing of two genes encoding RXO1-interacting proteins, revealed that *Os1PVOZ*, encoding a putative transcription factor, is a positive regulator of *Rxo1*-governed resistance and suggested that *OsATL6*, a putative E3 ubiquitin ligase is dispensable. Scanning the rice genome for putative PVOZ TF binding sites suggests that PVOZ TFs are involved in signal transduction cascades because of the enrichment in signaling molecules within PVOZ putative targets. Our data provide an overall picture of defense responses in maize, and suggest that *avrRxo1/Rxo1* induced defenses share several features identified in interactions between bacteria and dicot hosts. Our data also suggest that Rxo1 mediated resistance is mediated by phosphorylation cascades and early massive transcriptional cascades. OsPti1a and Os1PVOZ may provide signal amplification mechanisms that translate AvrRxo1 recognition into defense execution. Finally, using proteomic approaches may help in the identification of host proteins that mediate AvrRxo1 recognition. In the process, the biochemical and possible virulence functions of AvrRxo1 may be elucidated.

Co-immunoprecipitation experiments are needed to confirm RXO1 interaction with Os1PVOZ and other putative RXO1 interacting proteins. Quantitative experiments to assay for bacterial populations are also needed to strengthen the qualitative data obsverved with the loss of

the HR in *Os1pvoz* and *Ospti1a* silenced lines.Yeast-one-hybrid assays or in planta transient GUS reporter assays may show PVOZ interaction with the promoter of select putative targets. `Overall, microarray and RNAi data provide a platform for rationale driven and testable hypotheses.