Proc Indian Natn Sci Acad **75** No.1 pp. 49-68 (2009)

Attack and Defense in Xanthomonas-Rice Interactions

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(Received on 06 January 2009; Accepted on 16 February 2009)

Xanthomonas oryzae pv. *oryzae* (Xoo) is a gram negative phytopathogenic bacterium belonging to the genus xanthomonas, different members of which cause ~400 plant diseases. Xoo is the causal agent of Bacterial Blight (BB), a serious disease of rice. In this paper, we overview recent research on Xoo virulence functions and rice defense responses. The initial part of this review is an introduction to the rice-Xoo pathosystem and its advantages as a model for understanding the molecular basis of plant-pathogen interactions. This is followed by a comprehensive overview of the virulence functions of Xoo. The roles of adhesin like proteins, extracellular polysaccharide, lipopolysaccharide, pigment, protein secretion systems and their effectors, a cell-cell signaling system, iron uptake functions and some metabolic functions in the ability of this bacterium to cause disease are discussed. In the later part of the review, we outline some of the salient features of rice defense responses against Xoo infection. There are around 25 different rice loci that are known to impart resistance against different Xoo strains and six of them have been cloned to date. The characteristic features of these cloned resistance genes are summarized. Some of these resistance genes have been transferred, using molecular marker assisted selection, into the genetic background of commercially important rice varieties and the newly developed lines have been released for commercial cultivation.

Key Words: Plant Pathogenic Bacteria; Bacterial Virulence Mechanisms; Plant Defense Responses; Disease Resistance

Xanthomonas-Rice Pathosystem

Rice (*Oryza sativa* L.) is one of the major staple food crops of the world. There are two cultivated species of rice, namely *Oryza sativa* (cultivated in many parts of the world) and *Oryza glaberrima* (cultivated in West Africa). *Oryza sativa* includes two subspecies and, recently, the genomes of *Oryza sativa* spp. *indica* (long grained and non sticky) and *Oryza sativa* spp. *japonica* (short grained and sticky) have been sequenced [1-3]. The availability of the genome sequence, comparatively small genome size (~400Mb), presence of extensive genetic variation in the form of a large number of varieties and landraces, several dense genetic maps, a well developed transformation system, availability of thousands of insertion mutants and extensive synteny of genes with other members of the grass family have made rice a model monocotyledonous plant.

A number of bacteria, fungi, viruses and insects are known to affect rice. A better understanding of the mechanisms by which these pathogens and pests cause disease and the nature of the defense responses that are mounted by rice is important for development of new strategies for reducing yield losses. In this review, we focus on bacterial diseases of rice that are caused by members of the Xanthomonas group of plant pathogens with reference to the virulence strategies of the pathogens and the defense responses of the rice plant.

Members of the genus Xanthomonas cause diseases on ~400 plant species [4]. The xanthomonads are gram negative, flagellated, rod shaped, non-spore forming bacteria producing characteristic yellow colored pigments called xanthomonadins. Another distinct feature of this group is the production of copious amounts of extracellular polysaccharide. Two members of the genus Xanthomonas are rice pathogens. These are: *Xanthomonas oryzae* pv. *oryzae* (Xoo), which causes bacterial blight (BB) of rice and *Xanthomonas oryzae* pv. *oryzicola* (Xoc), which causes bacterial leaf streak (BLS) of rice. Although both Xoo and Xoc are foliar pathogens of rice, they have different tissue specificities. Xoo is a vascular pathogen, entering into the leaves either through hydathodes (natural openings which are concentrated at the leaf edges) or through wounds and multiplying in the xylem vessels (which are part of the circulatory system of the plant). In contrast, Xoc enters into the host either through the stomatal openings of leaves or through wounds and multiplies in the intercellular spaces within the parenchymatous tissue (which constitutes the major component of the green part of the leaf).

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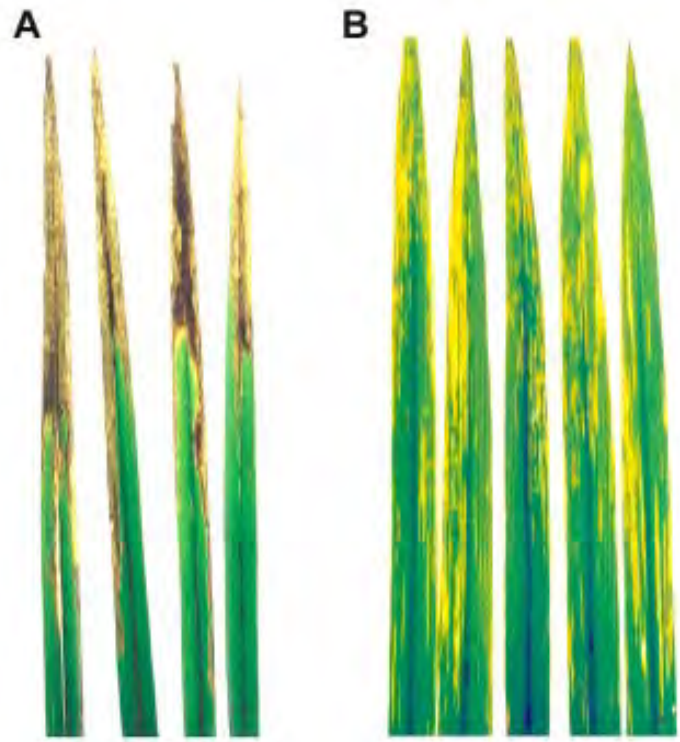

Fig. 1: *Diseases caused by Xanthomonas pathogens in rice. (A) Disease symptoms caused by* ***Xanthomonas oryzae*** *pv.* ***oryzae****. Infection starts from the leaf tip where hydathodes are located. The lesions spread down the leaf as the bacterium travels through the xylem vessels resulting in the drying symptoms that give a blighted appearance. (B) Disease symptoms caused by* ***Xanthomonas oryzae*** *pv.* ***oryzicola****. The intercellular spaces between rice leaf parenchyma cells are infected by this pathogen. A number of xylem vessels run parallel to each other, along the longitudinal axis of the leaf. The streak like lesions are due to the fact that this pathogen cannot infect xylem vessels and is hence restricted to interveinal regions.*

The characteristic symptoms of BB are yellow or brown lesions which begin at the tip and spread further down either one side or both sides or only through the midvein (Fig. 1a) until ultimately the entire leaf surface is affected. Ishiyama, working in Japan, first described the bacterial nature of the disease. BB is a very serious disease; the extent of yield loss depends on the severity and the stage of infection but can go upto 40%. Moreover, when infection happens in the seedling stage, the disease is lethal and results in wilting and death of the affected plants.

Secondary spread of Xoo occurs when the bacterium oozes out onto the surface of diseased leaves and the inoculum is splashed onto uninfected leaves (either on the same or on adjacent plants) by wind and rain. The conditions for this form of dispersal are found in India during the monsoon season, during which period the disease is especially prevalent in rice fields. The pathogen is believed to survive during the off season (when rice is not cultivated) on seeds, left over plant parts like leaf straw as well as wild varieties of rice or other members of the grass family which can serve as alternate hosts. In this manner the pathogen is able to persist and affect the rice crop in the next growing season. The genome sequences of three different strains of Xoo (from Japan, Korea and Philippines) have been recently reported [6-8]. The availability of the genome sequence of Xoo, well standardized tools for molecular genetic studies on this bacterium, plenty of known genetic variation (in the form of pathotypes that have been collected from different rice growing countries) and the advantages of rice as a genetic system make the Xoo-rice pathosystem an important model for studying host-pathogen interactions in plants.

BLS, which is caused by Xoc, was first described by Reinking in 1918 (in the Philippines) [9]. Later on Fang et al identified the causative bacterium and named the disease [10]. The characteristic symptoms of the disease are yellow or brown colored lesions which extend as streaks along the inter-veinal regions of the leaf (Figure 1B). The length and width of the streaks are variable. In the later stages of very severe infections, the different streaks can merge together to cause lesions that resemble those that occur due to BB. The mode of dissemination of this bacterium from plant to plant is similar to that of Xoo. The bacterium can survive on infected seeds and go on to infect seedlings. The genome sequence of a Xoc strain has been recently elucidated and is available in the public domain through the Comprehensive Microbial Resource of The Institute for Genomic Research (now a part of the J. Craig Venter Institute, USA). The availability of the genome sequences of two related pathogens (Xoo and Xoc) that infect the same host but with different tissue specificities

is an interesting feature of the Xanthomonas-rice pathosystem. Oligonucleotide microarrays for the genomes of both Xoo and Xoc are available on a single chip [11].

Virulence Strategies of Xanthomonas Pathogens of Rice

For successful pathogenesis, a bacterium must have functions for attaching and surviving on the plant surface, entry/penetration into the plant, suppression/evasion of host defense responses, multiplication within the plant, as well as dissemination to uninfected tissues, organs, and plants. We will review below the available literature on different virulence functions of Xoo and will include, wherever available, the relevant information on Xoc. Most researchers who study the Xanthomonas-rice interaction have concentrated their efforts on studies of Xoo as BB is a much more serious disease than BLS. Consequently, a lot more is known about the virulence strategies of Xoo as compared to Xoc.

Adhesin like Molecules

Attachment to the host surface is an important early step in the disease process. Bacterial pathogens have evolved cell surface localized proteins, called adhesins, which are required for recognition and binding to specific receptors on host surfaces. Ray et al have characterized a large (~110 KDa), outer membrane located Xoo protein called XadA (Xanthomonas adhesin like protein A) which is homologous to YadA, an adhesin of Yersinia pathogens of animals [12]. A large number of different sequence motifs are distributed throughout the length of the XadA protein and at least one of these motifs appears to be needed for generating the predicted beta-helical folds of this protein (G Aparna, R Sonti and R Sankaranarayanan, unpublished results). The XadA mutant of Xoo is compromised for virulence on rice in an assay which requires entry of the pathogen into rice leaves through the hydathodal openings [12]. This suggests an important role for the XadA protein in the early stages of infection i.e. attachment to the leaf surface and entry into the leaf. Consistent with this possibility, recent studies using confocal microscopy with a Green Fluorescent Protein tagged strain of Xoo indicate that XadA mutants are less efficient than the wild type strain in leaf colonization and entry through the hydathodal openings [13]. The *xadA* mutant retains normal levels of virulence when inoculation is by wounding. This method of inoculation involves direct deposition of Xoo into the xylem vessels and bypasses the hydathodal mode of entry into the plant. The result indicates that the XadA protein does not play a major role in promoting growth of Xoo within xylem vessels.

A paralog of XadA, called XadB (Xanthomonas Adhesin like Protein B) is encoded in the Xoo genome [6]. The *xadB* mutant also appears to be deficient for attachment to the leaf surface and entry through hydathodes and a *xadA xadB* double mutant is even more deficient than either of the single mutants [13]. The *xadB* as well as *xadA xadB* double mutants are as proficient for virulence as the wild type strain when inoculation is done by wounding. Again, this indicates that the XadB protein has a role in promoting leaf colonization and entry but is not required for growth within the xylem vessels. Orthologs of XadA and XadB are encoded in the genome of Xoc but their role in the virulence of this bacterium is yet to be determined. A readily identifiable difference between the various orthologs and paralogs of XadA in Xoo and Xoc lies in the number of sequence repeats, a feature which results in proteins of different lengths and, possibly, specificities (G Aparna, R Sonti and R Sankaranarayanan, unpublished results).

In addition to XadA and XadB, the Xoo and Xoc genomes encode homologs of a Yersinia adhesin-like protein called YapH (Yersinia autotransporter H). YapH mutants of Yersinia are virulence deficient in a *Caenorhabditis elegans* model of infection [14]. A mutation in the *yapH* gene of Xoo causes a deficiency in the early stages of leaf infection as well as for growth within the xylem vessels [13]. The Xoo and Xoc genomes also encode a set of genes for elaborating a Type IV pilus, which is a cell surface structure that is known to promote colonization of host surfaces in a number of pathogenic bacteria. Mutations in the Xoo *pilQ* gene, which is predicted to be involved in type IV pilus assembly, are virulence deficient upon wound inoculation [13, 15]. However, *pilQ* mutants are as proficient as the wild type Xoo strain in leaf colonization/entry. This indicates that the *pilQ* gene, and hence type IV pilus, plays an important role in promoting growth within the xylem vessels but does not play an important role in the early stages of leaf colonization/entry. Overall, these studies indicate that multiple adhesin-like functions are involved in promoting virulence of Xoo with some of these functions being more important at particular stages of the disease process than at others. Several virulence deficient mutants of Xoc that were isolated following a transposon mutagenesis screen have been found to be defective in genes (*pilQ*, *pilM*, *pilT*, *pilY1*, and *pilZ*) which are involved in assembly of Type IV pilus [16].

Extracellular Polysaccharide (EPS)

The presence of copious amount of EPS is a characteristic feature of the genus *Xanthomonas* and is responsible for the mucoid appearance of the colonies of these bacteria. The EPS from *Xanthomonas campestris* pv.

campestris (Xcc; a pathogen of plants like cabbage and caulifower) is called xanthan gum and has a large number of applications in the food industry. The EPS of Xcc is composed of a repeated unit of pentameric sugar moieties comprising of two glucose, two mannose and one glucuronic acid molecule [17]. The EPS biosynthetic gene cluster of Xoo (called the *gum* cluster) exhibits a very high level of sequence identity (80-90%) to the EPS gene cluster of Xcc, suggesting that the EPS of these two bacteria is likely to have a similar composition and structure. The *gum* gene cluster of Xoo contains 14 *gum* (*B* to *N*) genes [6] and constitutes a polycistronic operon [18, 19]. Mutations in this cluster have been shown to result in virulence deficiency [20, 21]. However, the exact role of EPS in virulence of Xoo has not been determined. A recent study suggests that EPS of Xcc may have a role in suppression of host defense responses through its ability to sequester calcium [22].

Lipopolysaccharide

Lipopolysaccharide (LPS) is an important constituent of the outer membrane of gram-negative bacteria. It is composed of a lipid portion called Lipid A, which is embedded in the outer membrane, an inner and outer oligosaccharide core and a polysaccharide chain (commonly known as O-antigen) that is exposed on the bacterial cell surface. The O-antigen is hyper variable in different strains of animal pathogenic bacteria and the variation is due to the kinds of sugars present, their arrangement and the linkage between different oligosaccharide units [23]. LPS has been shown to be required for the virulence of several animal and plant pathogenic bacteria [24-27]. The role of LPS in virulence may derive from its ability to protect the bacterium against various host derived anti-microbial compounds. Interestingly, LPS has also been shown to act as a Pathogen Associated Molecular Pattern which is recognized by the host to mount potent defense responses in different animals and plants [28, 29].

Dharmapuri *et al.* have identified a Xoo locus with atypical G+C content that is required for the production of LPS [25]. Mutations in three genes that were identified at this locus were shown to result in a deficiency in production of LPS as well as virulence. In a subsequent study, Patil and Sonti have further characterized this genomic locus and identified six open reading frames (ORFs) that are encoded by it [30]. All of these ORFs have atypical G+C content and altered codon usage pattern, as compared to other genes in the Xoo genome. These features suggest that the Xoo *lps* locus has been acquired by horizontal gene transfer, albeit from an as yet unidentified source. This locus is present in a number of Xoo strains from India and other Asian countries but is absent from one Indian Xoo strain (called BXO8) and one Nepalese strain (called Nepal 624). The *lps* gene cluster of the BXO8 strain has also been characterized and found to encode 15 genes [31], 14 of which are orthologous to genes in the *lps* locus of *Xanthomonas axonopodis* pv. *citri* (Xac; a pathogen of citrus plants). Mutations in several genes of the BXO8 *lps* gene cluster have been shown to result in LPS as well as virulence deficiency (MG Anil, Prabhu Patil and R Sonti, unpublished data). The Xoc pathogen seems to have a hybrid *lps* gene cluster; one part of this gene cluster (comprising of nine genes) is orthologous to the *lps* gene clusters of the BXO8 and Xac strains while the remaining seven genes are homologous to genes in phylogenetically distant bacteria [31]. These results have provided the first evidence of hyper variation at *lps* biosynthesis gene clusters within different strains of phytopathogenic bacteria and are suggestive of the occurrence of multiple horizontal gene transfer events at this locus in xanthomonad pathogens of rice. This kind of hyper variation and horizontal gene transfer at *lps* biosynthetic gene clusters has been detected earlier in animal pathogenic bacteria, where it has been ascribed to help in evasion of the host immune response [32]. It is tempting to speculate that the variation in *lps* biosynthetic gene clusters in plant pathogenic bacteria might also help in evasion of the host defense response. The *lps* deficient mutants of the BXO1 and BXO8 strains [25; MG Anil, Prabhu Patil and R Sonti, unpublished data] are also deficient for EPS production. The role of these *lps* genes in promoting EPS synthesis is not known. In a recent study, a mutation in the *lps* biosynthetic gene cluster of Xoc has been shown to result in virulence deficiency on rice [16].

Pigment

Xanthomonadins are brominated, outer membrane located aryl polyene pigments. Xanthomonadin deficient mutants are proficient for virulence but are hypersensitive to photooxidative stress [33]. It has been postulated that xanthomonadin may have a role in promoting survival of the bacterium on leaf surfaces (prior to entry into the plant) by providing protection against photooxidative damage. Indeed, xanthomonadin deficient mutants of Xcc have been shown to be deficient at survival on leaf surfaces and to be virulence deficient in an assay which requires their survival on leaf surfaces prior to entry into the plant [34]. Genes involved in pigment biosynthesis have been cloned and characterized from Xoo [35-36]. The results suggest that the polyene tail of xanthomonadin may be derived from a polyketide pathway and that the aryl ring may be derived from the shikimate pathway. A putative membrane transporter, which is homologous to multidrug efflux proteins, has been shown to be required for outer membrane localization of xanthomonadin.

Protein Secretion Systems and their Effectors

A number of different secretion systems are employed by gram-negative bacteria to transport proteins to the extracellular milieu [37]. Depending upon the manner in which the proteins are secreted through the inner and outer membranes and the composition of the secretion apparatus, the secretion systems have been categorized into at least six major groups. Detailed overviews of these secretion systems and their secreted proteins in gram-negative plant pathogenic bacteria are included in several reviews [37-42]. As indicated below, three different Xoo protein secretion systems have been studied in detail.

Type I Secretion and AvrXa21 Activity

The Type I secretion system (T1SS) is involved in exporting proteins across the two bacterial membranes (inner and outer) to the extracellular milieu [43]. Three genes namely *raxA*, *raxB* and *raxC* encode the components of a T1SS of Xoo. RaxA is homologous to a Membrane Fusion Protein (MFP; spans the periplasm and extends upto the outer membrane), RaxB is similar to a ABC transporter (provides energy for transport by ATP hydrolysis and forms a channel across the inner membrane) and RaxC is similar to the *E. coli* outer membrane protein TolC (which completes the channel and forms the point of exit of the type 1 substrate) [44]. These genes are named *rax* because they were isolated as genes **r**equired for **A**vr**X**a21 activity. Xoo strains having AvrXa21 activity are avirulent on rice lines containing the resistance gene, *Xa21*, but are virulent on rice lines that do not have *Xa21*. Mutations in any of these *rax* genes result in loss of *avrXa21* activity and lead to a 100-1000 fold increase in bacterial survival within *Xa21* containing rice lines but do not affect virulence on rice lines lacking *Xa21* [44]. Mutations in three other genes have also been shown to affect AvrXa21 activity. These genes are predicted to encode functions involved in sulfur metabolism; specifically *raxP* encodes ATP sulfurylase, *raxQ* encodes adenosine phosphosulfate kinase and *raxST* shows similarity to sulfotransferases [44, 45]. It is speculated that these genes might be involved in sulfation of AvrXa21 in Xoo and that this modification might play a critical role in recognition by the Xa21 protein. The *raxAB* and *ST* genes are present in an operon. Two additional *rax* genes are found to be present (probably in an operon), downstream to the *raxSTAB* operon [46]. Out of these, *raxR* shows similarity to OmpR family of response regulators and *raxH* is homologous to histidine protein kinases. These two categories of proteins comprise two component regulatory systems that are involved in modulating bacterial gene expression in response to environmental cues. The Xoo strains having a null mutation in either or both of these genes are compromised for AvrXa21 activity. Furthermore, the authors have shown that expression of the *raxSTAB* operon is regulated by *raxR/raxH*.

These studies suggest that a bacterial T1SS is required to secrete AvrXa21 into the extracellular medium where it is recognized by Xa21 expressing rice lines to induce resistance. In order to test this hypothesis, a novel plant bioassay has been developed [47]. In this assay, rice leaves are pretreated with the cell free supernatants of different Xoo strains that either possess or lack AvrXa21 activity followed by subsequent inoculation with wild type Xoo. Pretreatment of leaves of rice plants containing the *Xa21* resistance gene with the culture supernatant of wild type Xoo (AvrXa21$^+$) prevented disease formation whereas the culture supernatants of AvrXa21$^-$ strains (*raxST*$^-$, *raxA*$^-$, *raxB*$^-$, *raxC*$^-$, *raxP*$^-$ and *raxQ*$^-$) were not able to prevent infection by subsequent inoculation with Xoo [47]. Using reverse phase HPLC, two AvrXa21 activity containing fractions have been isolated from the culture supernatant of wild type Xoo. Preliminary analysis suggests that AvrXa21 may be a small peptide/s [47]. Most importantly, the AvrXa21 molecule has been suggested to promote cell density dependent gene expression (quorum sensing) in Xoo. It has been proposed that AvrXa21 is a novel signaling factor that is used in cell-cell communication in Xoo, particularly for regulation of virulence gene expression. It appears that rice plants containing the *Xa21* resistance gene are able to use the AvrXa21 molecule as a cue for the presence of Xoo and activate *Xa21* dependent resistance responses.

Lee *et al*. have identified a Xoo two component regulatory system named *phoP*/phoQ which, in association with the *raxR*/*raxH* system, is involved in regulation of AvrXa21 production [48]. The PhoP protein functions as a transcriptional activator and the PhoQ acts as a sensor protein/histidine kinase. The *phoQ* mutant of Xoo is capable of causing disease on *Xa21* containing rice lines, suggesting its involvement in the production of AvrXa21. The *phoP* mutant is virulence deficient, even on rice lines that lack the *Xa21* gene, indicating that PhoP is involved in regulating the production of certain basic virulence factors.

Type II Protein Secretion System and its Effectors

The Type II secretion system (T2SS) secretes proteins from the cytosol to the extracellular milieu in a two-step process. In the first step, the secreted proteins are translocated across the cytoplasmic membrane using either the sec-dependent pathway [49] or Twin-Arginine (Tat) pathway [50], depending on the nature of the signal peptide. In the second step, the proteins are secreted from the periplasm across the outer membrane to the extracellular medium, by the T2SS apparatus whose

components are encoded by approximately 12 to 16 genes [51, 52].

A number of gram-negative bacterial plant pathogens have been shown to encode a T2SS. These include representatives from major groups of bacterial plant pathogens including *Erwinia*, *Pseudomonas*, *Xanthomonas*, *Xylella*, and *Ralstonia*. A notable exception is *Agrobacterium tumefaciens*, which does not appear to encode a T2SS [53, 54]. *Xcc* and *Xac* appear to encode two complete T2SS gene clusters, called *xcs* and *xps*, in their genomes [55]. T2SS is an important virulence factor for a number of phytopathogenic bacteria including several Xanthomonads [41]. Mutations in the T2SS gene cluster of Xoo result in virulence deficiency [56]. A number of proteins have been shown to be secreted through the T2SS in laboratory grown cultures of Xoo. The interesting feature is that these proteins appear to function as plant cell wall degrading enzymes. These proteins include a cellulase (ClsA), cellobiosidase (CbsA), xylanase (XynB) and lipase/esterase (LipA) [57, 58]. Mutations in genes for LipA, XynB or ClsA of Xoo cause partial loss of virulence, while *lipA xynB* and *lipA clsA* double mutants have much more severe effects on virulence [57, 58]. A CbsA deficient mutant of Xoo is severely virulence deficient [57]. These results indicate that mutations in individual T2SS secreted proteins have varying effects on virulence with some of them being much more important for virulence than others. Also, it appears that there is a functional redundancy amongst T2SS secreted proteins with regard to their role in virulence as some of the double mutants are much more severely virulence deficient than the single mutants. The Xoo genome encodes a large number of cell wall degrading enzymes including multiple cellulase and xylanase like enzymes as well as a pectinase [6]. One cellulase, called EglXoB (different from ClsA), appears to be expressed only during *in planta* growth of Xoo. Mutations in *eglXoB* cause loss of Xoo virulence [59]. This study raises the interesting possibility that some of the plant cell wall degrading enzymes that are encoded in its genome are synthesized by Xoo only during growth within rice plants.

Type III Protein Secretion System and its Effectors

Type III protein secretion system (T3SS) is found in a large number of animal and plant pathogenic bacteria. The T3SS forms a needle like apparatus through which it delivers proteins directly into the host cells [38-40]. T3SS was initially identified in animal pathogenic bacteria where it was shown to be an important virulence determinant delivering virulence factors into host cells. It has been characterized from a number of phytopathogenic bacteria including several xanthomonads and demonstrated to play a critical role in plant-pathogen interactions. The genes encoding the T3SS are present in a cluster in xanthomonads [55, 60-63]. Mutations in this gene cluster can lead to virulence deficiency on susceptible host plants and an inability to provoke hypersensitive response (HR; a plant programmed cell death response that is associated with a resistance reaction) in resistant varieties of host plants and on non-host plants. The T3SS gene cluster of plant pathogenic bacteria is also called *hrp* (hypersensitive response and pathogenicity) cluster. The genes that are conserved amid the T3SS gene clusters of plant and animal pathogenic bacteria have been further classified as *hrc* (**h**ypersensitive **r**esponse **c**onserved) genes whereas those genes which are present only in the phytopathogenic bacteria are called *hrp* genes [64]. Genes that encode the T3SS components and T3SS secreted proteins are generally expressed only when the bacterium comes into contact with the host although certain nutrient limiting minimal media that mimic host conditions also trigger the expression of these genes [65, 66].

In Xanthomonads, various studies have revealed that the HrpX and HrpG proteins are involved in regulation of the expression of *hrp* genes [66-70]. The HrpG protein is a transcriptional activator, belonging to the OmpR family of two-component response regulators. It acts as a response regulator which is phosphorylated through the action of an as yet unidentified kinase protein. Phosphorylation of HrpG leads to activation of the expression of *hrp*X (which encodes a transcriptional activator). The expression of HrpX leads to expression of genes that encode components of T3SS and those that encode T3SS secreted proteins. The HrpX regulated genes have a consensus sequence in their promoter region which is known as a plant inducible promoter (PIP) box. The presence of the PIP box has been used to identify the predicted HrpX regulon in several xanthomonads whose genome sequences are available. In Xoo, the role of the consensus PIP box sequences (TTCGC-N_{15}-TTCGC) in HrpX dependent regulation has been examined [71]. This study has revealed that base substitutions in the consensus PIP box do affect the expression of *hrp* genes but do not completely abolish their expression. Even two base pair substitutions from the consensus allowed HrpX dependent gene expression, albeit at a reduced level as compared to the wild type level of expression [71]. These results suggest that even imperfect PIP box containing genes can be regulated by HrpX. At least two genes have been identified in Xoo, which have imperfect PIP boxes and are regulated by HrpX. Furutani *et al.* have shown that HrpX of Xoo can not only upregulate the expression of the genes for T3SS and its secreted proteins but can also upregulate the

expression of several T2SS secreted proteins, presumably because these are also required only during growth within the rice plant [72].

The *hrp* gene cluster of Xoo has been cloned by Zhu *et al.* [63]. Mutations in the *hrpA*, *hrpB*, *hrpC* or *hrpD* genes of the *hrp* cluster cause virulence deficiency and an inability to induce HR in resistant rice lines. Mutations in two genes that are adjacent to the *hrp* cluster, called *hpa1* and *hpa2* (for hrp associated), also cause deficiency in virulence and the ability to induce HR. The HrpF is a T3SS secreted protein that is predicted to function as a translocon in *Xanthomonas campestris* pv. *vesicatoria* (Xcv; a pathogen of tomato plants) [73]. Mutations in the *hrpF* gene of Xcv cause virulence deficiency and an inability to induce HR. A *hrpF* deficient mutant of Xoo is severely virulence deficient but it is able to induce HR in rice cultivars containing either *Xa7* or *Xa10* resistance genes [74]. The authors suggest that secretion through T3SS is reduced but not eliminated in the *hrpF* mutants of Xoo and that HR is induced even with reduced translocation of T3SS effectors.

Zou *et al.* have characterized the T3SS gene cluster of Xoc and reported that it is highly homologous to such genes in other xanthomonads including Xoo with some minor variations [75]. Mutations in the T3SS gene cluster as well as in the *hrpX* and *hrpG* genes cause loss of virulence and an inability to induce HR on non-host tobacco plants [16, 75]. The Hpa1 protein has also been characterized from Xoc. Its expression is found to be regulated by HrpX and is expressed only in plant mimic medium. The *Hpa1* gene has been over-expressed in *Escherichia coli* and the purified protein has been shown to induce the HR when infiltrated into tobacco leaves.

What are the proteins that are secreted through the T3SS of Xoo? Proteins that are encoded by a multi-gene family, called the *avrBs3* family (based on the name of the prototype which was first identified in Xcv), have been shown to be secreted through the T3SS of Xoo. The members of the *avrBs3* family share 90-97% sequence identity at the amino acid level and exhibit the presence of a variable number (13.5-25.5) of nearly identical 34 amino acid repeats at their central domain as well as Nuclear Localization Signals (NLSs) and an Acidic transcriptional Activation Domain (AAD) at their C-terminal region [76]. Mutational studies on two members of this gene family in Xoo, called *avrXa7* and *avrXa10*, have demonstrated that the AAD and the NLS of these proteins is required for eliciting HR [77] on non-host plants. Four different members of this gene family have been cloned from a Philippine isolate of this pathogen [78, 79]. The genome sequence of a Korean strain of Xoo (KACC 10331) has revealed the presence of eight homologues of *avrBs3* [6]. The *avrXa7* gene of Xoo has been shown to encode a T3SS dependent, nuclear localized, double stranded DNA binding protein [80] and a mutation in this gene causes a substantial loss of virulence. Mutations in several other members of the *avrBs3* family of Xoo have been shown to cause only a partial loss of virulence [81] suggesting that some members of this gene family are more important for virulence than others.

What is the role of these T3SS secreted proteins in virulence of Xoo? A Xoo protein called PthXo1, which is a member of the avrBs3 family of proteins, appears to be translocated into rice cells wherein it serves to upregulate the expression of specific rice genes [82]. In particular, the expression of the rice gene Os*MtN3* (which encodes a protein that is related to nodulin proteins of legumes) is upregulated more than 1000 fold upon infection with PXO99, a Xoo isolate from the Philippines. Xoo mutants that are defective in either the T3SS or in PthXo1 are unable to upregulate the expression of *OsMtN3* and are virulence deficient. It appears that increased expression of *OsMtN3* is essential for growth of Xoo within rice cells. The exact manner in which the increased expression of *OsMtN3* promotes Xoo growth within the host remains to be established but it is clear that OsMtN3 represents the prototype of an important category of proteins that are being referred to as host susceptibility factors. Recently, the expression of two other rice genes has also been found to be upregulated during Xoo infection. One of these genes (*OsTFX1*) shows homology with genes encoding members of the bZIP family of transcription factors whereas the other gene (*OsTFIIAγ1*) exhibits homology with a small subunit of transcription factor TFIIA, which is a part of the rice transcription machinery [83]. The increased expression of these rice genes following infection by Xoo has been shown to require functionality of T3SS as well as the translocation of two other members of the AvrBs3 family of transcription activators called PthXo6 (which is required for upregulation of *OsTFX1* expression) and PthXo7 (required for the upregulation of the expression of *OsTFIIAγ1*). Several members of the *avrBs3* family of T3SS effectors are also present in Xoc but their role in the Xoc-rice interaction has not yet been established.

The Xoo T3SS also secretes a number of proteins that do not belong to the *avrBs3* family. Using a combination of bioinformatics and biochemical analyses, 16 such Xoo proteins have been shown to be translocated into plant cells through the T3SS [84]. Although a few of these proteins are specific to Xoo, the majority are widely distributed amongst the xanthomonads. The functions of these proteins are not known. It is likely

that at least some of them are involved in suppression of rice innate immunity [57].

An Xoc avirulence gene, *avrRxo1*, which is required for eliciting the non host resistance response on maize plants carrying the *Rxo1* gene has been identified [85]. This gene does not show significant homology with any available proteins in the databases but contains a predicted active site motif of eukaryotic cysteine (thiol) proteases and also a P-loop (ATP/GTP binding site motif A) motif. Xoo cells overexpressing Xoc *avrXo1* gene can effectively secrete this protein into maize cells in a T3SS dependent manner. The transient expression of AvrRxo1 induces Rxo1 protein mediated cell death responses in maize [85]. Transgenic rice plants overexpressing the maize *Rxo1* gene have been found to be resistant to Xoc infection [86].

In an interesting experiment, coinfiltration of wild type Xoc into intercellular spaces of rice leaves along with a Xoo strain expressing AvrXa7 prevented the induction of a HR in a rice cultivar containing the *Xa7* resistance gene [87]. A T3SS deficient mutant of Xoc was unable to suppress the HR that is induced by *avrXa7* containing Xoo strains. These results suggest that Xoc has the ability to hinder resistance gene mediated defense response in rice and that this ability is dependent on the presence of a functional T3SS. The Xoc T3SS secreted proteins that are involved in suppression of rice defense responses remain to be identified. It is pertinent to note that, in spite of extensive screening, there are no known sources of resistance in rice against infection by Xoc. It is possible that this might be because Xoc has a very efficient arsenal of T3SS secreted proteins for suppressing rice defense responses.

What is the explanation for the dual role of T3SS secreted proteins in virulence and in inducing host resistance? It appears that T3SS secreted proteins are being translocated into rice cells where they serve very important functions in promoting pathogenesis, such as the upregulation of host susceptibility factors and suppression of innate immunity. It seems that some of these T3SS secreted proteins are also being recognized by the rice plant as a cue for the presence of the pathogen and being used to mount defense responses. The recognition of these secreted proteins may be occurring in very specific interactions that can only be performed by rice lines that contain particular genes called '*Resistance*' genes. The characteristics of several cloned rice '*Resistance*' genes are described later on in this review.

Cell-Cell Signaling System

In order to cope with the challenging environment within host cells, proper communication between bacterial cells is a requisite for successful pathogenesis. One signaling molecule that xanthomonads use for this purpose is called diffusible signaling factor (DSF). The production and sensing of DSF is encoded by the *rpf* (regulation of pathogenicity factors) gene cluster, harboring *rpfA–rpfI* genes. This gene cluster has been first identified and characterized in Xcc [88]. The chemical structure of Xcc DSF has been characterized as cis-11-methyl-2-dodecenoic acid, an α, β unsaturated fatty acid [89]. A complete overview of the Rpf/DSF signaling pathway and its role in plant-pathogen interactions is provided in several recent reviews [90, 91].

The genomic organization of the *rpf* gene cluster in Xoo is similar to that of Xcc and mutations in *rpf* genes cause virulence deficiency in both bacteria. However, some of the phenotypes associated with *rpf* deficiency are different in Xoo and Xcc. For example, the Xoo *rpfF* mutant is proficient for EPS and extracellular enzyme production whereas the Xcc *rpf* mutant is deficient for both [92-94]. The *rpfF* Xoo mutant bacteria overproduce siderophores (low molecular weight ferric iron chelators) and exhibit a defect in expressing tetracycline resistance, both phenotypes are indicative of iron starvation [93]. The *rpfF* Xoo mutants exhibit a growth defect in iron-depleted medium. The authors, using a detached leaf assay, have demonstrated that exogenous iron supplementation promotes growth of *rpfF* mutants in rice leaves. These results suggest that the virulence deficiency of the Xoo *rpfF* mutant might be due to its inability to source sufficient iron during in planta growth.

In addition, the *rpfC* gene (which is involved in sensing DSF) is also required for Xoo virulence. The *rpfC* Xoo mutants exhibit reduced EPS but no change in extracellular enzyme production [94]. This is again in contrast to the phenotype of Xcc *rpfC* mutants which are deficient for production of EPS as well as extracellular enzymes. The chemical structure of Xoo DSF is not known but it is likely to be similar to that of the DSF of Xcc. Further research has to be carried out to identify the *rpf* regulon of Xoo and to characterize the role of individual genes of this regulon in virulence. DSF has also been linked with optimal virulence of Xoc, as a mutation in a gene involved in the DSF regulatory pathway causes reduced virulence on rice [16].

Iron and Zinc Uptake Functions

Iron plays a crucial role in the redox reactions of intermediary metabolism and acts as a co-factor of several important enzymes. However, the availability of iron is limited by its low solubility in aerobic conditions at neutral and alkaline pH. Inside host tissues, iron is further sequestered by iron binding proteins, reducing the availability of iron molecules even further for pathogenic bacteria. Under such iron limiting conditions,

many bacteria produce siderophores. Studies have shown that siderophore-mediated iron uptake is critical for the ability of several plant pathogenic bacteria to grow within their hosts [95]. Recently, the siderophore biosynthetic gene cluster of Xoo has been identified through bioinformatics approaches. Mutations in this gene cluster cause a total loss of siderophore production by Xoo but do not affect virulence (Alok Pandey and Ramesh Sonti, unpublished results). However, a mutation in the Xoo homolog of the *feoB* gene (encodes the major bacterial ferrous transporter) causes a total loss of virulence on rice. These studies clearly show that ferrous uptake through FeoB is much more important for Xoo virulence than siderophore mediated ferric iron uptake.

The presence of excess iron inside cells is lethal as it leads to overproduction of reactive oxygen species. Hence, the expression of iron uptake functions needs to be tightly controlled. In several bacteria, this regulation is done by the Fur (Ferric Uptake Regulator) protein. Homologs of the *fur* gene have been identified in a number of xanthomonads [96]. The *fur* mutant of Xoo exhibits constitutive production of siderophores and grows very poorly within rice leaves [97]. Exogenous supplementation of ascorbic acid (an anti-oxidant) rescues the in planta growth deficiency of the Xoo *fur* mutant. It appears that the in planta growth deficiency associated with the Xoo *fur* mutant is, at least in part, due to its inability to combat oxidative stress during infection. Further studies on the *fur* regulon of Xoo are needed to understand how the Fur protein controls iron uptake, promotes resistance to oxidative stress and growth within the host. A mutation in the zinc uptake regulator (*zur*) gene of Xoo has been characterized [98]. The *zur* mutant of Xoo is virulence deficient, unable to grow on zinc or iron supplemented medium, hypersensitive to hydrogen peroxide and produces lesser catalase and EPS than wild type.

Metabolism

Mutations that affect certain metabolic pathways have been reported to cause loss of virulence in Xoo. A transposon insertion in the *purH* gene (which encodes a bifunctional purine biosynthesis protein having formyltransferase and hydrolase activities) results in virulence deficiency and purine auxotrophy [99]. Mutations in the *purD* gene which encodes a phosphoribosylamine-glycine ligase that is involved in purine biosynthesis also cause loss of virulence and purine auxotrophy [100]. These results suggest that Xoo might be unable to source purine from host tissues. A Xoo mutant that is defective in the *aroE* gene, which encodes shikimate dehydrogenase, is an aromatic amino acid auxotroph and is deficient for virulence as well as xanthomonadin pigment production [36]. The virulence deficiency of the *aroE* mutant is likely to be due to the aromatic amino acid auxotrophy as the vast majority of xanthomonadin deficient mutants are virulence proficient. The virulence deficiency of the *aroE* mutant indicates that Xoo cannot source one or the other aromatic amino acids from the host while the pigment deficiency of this mutant suggests that the aryl ring in the pigment molecule is derived from the shikimate pathway [36].

A transposon induced Xoo mutant has been isolated which contains a mutation in the phosphoglucose isomerase (*pgi*) gene. The mutant demonstrates reduced virulence on rice [101]. Pgi is involved in the gluconeogenesis pathway wherein it catalyses the reversible isomerisation of glucose 6 phosphate and fructose 6 phosphate. The Xoo *pgi* mutant is deficient for growth in mimimal medium containing fructose or xylose as the sole carbon source suggesting that the virulence deficiency associated with the *pgi* mutant may be due to an inability to utilize either xylose or fructose as a sole carbon source. Tsuge et al have reported a Xoo mutant which is deficient in utilizing glucose as a carbon source but still exhibits full virulence on rice [102]. This result suggests that Xoo is not solely dependent on glucose as a carbon source during its growth within rice leaves.

Phytic acid (inositol hexaphosphate) is considered to be a major storage form of total phosphorus in cereals and legumes. Phytases are enzymes involved in hydrolysis of phytic acid into less phosphorylated myo-inositol derivatives. Mutations in the Xoo *phyA* (putative phytase A; homologous to a Bacillus Phytase) gene cause virulence deficiency and an inability to use phytic acid as a sole phosphate source [103]. In a detached leaf assay, exogenous supplementation with phosphate has been shown to promote growth of the *phyA* mutant in rice leaves. This result suggests that the virulence deficiency associated with the *phyA* mutant is due to its inability to use phytic acid as a phosphate source.

Some metabolic defects have also been found to affect virulence of Xoc on rice. Mutations in the Xoc genes involved in carbohydrate metabolism such as *pgk*, encoding phosphoglycerate kinase; *gap*, encoding type 1 glyceraldehyde-3-phosphate dehydrogenase and *ppsA*, encoding phosphoenolpyruvate synthase A, have been found to impart moderate to drastic reduction of virulence on rice [16]. Furthermore, mutant Xoc strains that are defective either in fatty acid biosynthesis or the prephenate pathway for aromatic amino acid biosynthesis have been found to be partially virulence deficient on rice [16].

Rice Defense Responses

Plants and their bacterial pathogens have been coevolving during the millions of years of their coexistence. During this process, plants have evolved to perceive the presence of pathogens and mount defense responses to evade further infection [104-108]. Plants can recognize a number of pathogen associated molecular patterns (PAMPs) such as flagellin [109], lipopolysaccharides [110], cold shock protein [111], elongation factor Tu [112, 113], plant cell wall degrading enzymes and cell wall degradation products [57, 114, 115] or strain specific molecules such as the avirulence factors [116]. On the perception of any of these factors, plants induce production of phenolics, phytoalexins, deposition of lignin and callose on plant cell walls, sudden increase in the production of reactive oxygen species (oxidative burst), alkalization of cytoplasm due to ion fluxes across plasma membrane, programmed cell death, etc. which contribute towards arresting the growth of the pathogen [106, 114, 115, 117, 118]. These defense responses are part of the innate immune system of the plant and serve to protect plants against most potential pathogens [119, 120]. The recognition of PAMPs by the innate immune system of plants is believed to occur at the plant cell surface through the action of receptors that are related to proteins which do a similar function in the innate immune system of animal cells. However, very little is known about the mechanisms by which plant innate immunity is elaborated and its mode of action.

Plant pathogens have the capacity to suppress these innate immune responses of their hosts. In fact, this ability of plant pathogens to suppress innate immunity appears to be a precondition for their ability to cause disease [57]. Suppression of innate immunity often occurs through the action of pathogen effector proteins that are secreted directly into plant cells through the T3SS apparatus [57, 106, 121-125]. It further appears that there is a second level of interaction between plants and their pathogens, wherein the host has evolved certain '*R*' (resistance) genes whose products recognize T3SS effectors either directly or indirectly [119, 120, 126-128] resulting in a more specific secondary defense response. The '*R*' gene mediated resistance is effective against certain races of the pathogen and not against others. Presumably, this occurs because the product of the '*R*' gene allows the plant to recognize certain pathogen strain specific T3SS effectors (called avirulence factors which are encoded by *avr* genes) either directly or indirectly to mount a defense response. The products of most '*R*' genes are localized in the cytoplasm and act within plant cells. The extent of overlap and the points at which innate and 'R' gene immunity intersect are not clear. However, '*R*' genes themselves are quite heterogenous. As discussed below, a few '*R*' genes like *xa13* of rice are alleles which prevent upregulation of host susceptibility factors by the pathogen while others such as *Xa21* are now being considered to be a form of plant innate immunity as they are related to proteins that function in innate immunity. These genes would therefore have a mode of action that is different from those of typical '*R*' genes.

We provide below a brief overview of certain important features of rice defense responses against infection by xanthomonads. As is the case with studies on bacterial virulence functions, a lot more research has been done on rice defense responses against Xoo infection. In comparison, very little work has been done to understand rice defense responses against infection by Xoc.

Interaction between plants and their pathogenic microbes can lead to either susceptibility or resistance. The susceptible interactions are called compatible interactions while the resistant interactions are called incompatible interactions. The ultra structural and biochemical changes that take place in rice tissues upon infection by Xoo have been characterized. In the incompatible (i. e. resistant) interaction, Xoo cells can be detected in the xylem vessels but their shape becomes distorted and they are found to be enclosed in a fibrillar material within three days after infection whereas in a compatible (susceptible) interaction the cells appear normal (and are not covered by the fibrillar material) even 20 days after infection [129, 130]. Detailed electron microscopic studies have revealed changes in the cytoplasm of xylem parenchyma cells within 24 hr and thickening of the secondary cell wall of xylem vessels with a reduction in the diameter of the pits (that connect adjacent xylem vessels) within 48 hr of inoculation with a Xoo strain that gives rise to an incompatible reaction and not with a Xoo strain that gives a compatible interaction [131]. Interestingly, a certain amount of thickening of xylem secondary cell wall and reduction in pit diameter are also observed even in a compatible interaction with older rice plants which is consistent with their being intrinsically more resistant to Xoo infection.

Peroxidase enzymes have been implicated as key players in plant defense responses wherein they participate in diverse physiological functions such as lignification of the plant cell wall, phenolic oxidation, cross linking of polysaccharides as well as monomers of extensin proteins in plant cell walls, generation of oxidative free radicals, etc. During an incompatible interaction between rice and Xoo, increased activities of a cationic and two anionic isoforms of extracellular peroxidases have been reported [132, 133]. The timing of the increase in peroxidase activities correlates with

that of the deposition of lignin and phenolics in the plant cell wall, a decline in bacterial multiplication and onset of the HR. This is suggestive of an important role for peroxidases in rice defense responses against Xoo infection. The Xoo induced rice cationic peroxidase (PO-C1) protein has been purified [134] and the gene has been cloned [131]. The PO-C1 protein demonstrates a high similarity with pathogen-induced peroxidases in other plants. The expression of *PO-C1* is induced within 12 hr of an incompatible interaction with Xoo and the protein is accumulated in the xylem parenchyma cells and in the lumen and walls of xylem vessels [131, 134]. The *PO-C1* gene is induced only during the incompatible interaction with Xoo and not by wounding or during normal development [131]. Based upon its involvement in the thickening of secondary cell walls, accumulation in the cell wall during lignification and an ability to utilize coniferyl alcohol (a precursor of lignin) as a substrate, the PO-C1 protein has been hypothesized to function as a lignin peroxidase which promotes lignification by cross linking lignin precursors such as coniferyl alcohol [131, 132, 135]. Increased lignin deposition has in fact been reported during the incompatible interaction between rice and Xoo [135], particularly in association with an induction of HR. Lignin is a large, polymeric noncarbohydrate constituent of plant cell walls and is hydrophobic and aromatic in nature. It plays a crucial role in preventing the spread of pathogens by forming a structural barrier. The free radical intermediates and superoxides produced during polymerization of lignin also serve to inhibit the growth of both bacterial and fungal pathogens.

Young *et al.* have reported that rice phospholipase D (PLD) localizes to the plasma membrane during the incompatible interaction of rice with Xoo [136]. The PLD enzyme hydrolyzes phospholipids in the plant cell membrane to generate phosphatidic acid, which in turn can act as a second messenger. The PLD enzyme has been shown to play a critical role in cellular signaling in mammalian systems and an increase in the activity of PLD has been reported in plant cells that are undergoing a HR. An increase in the expression of rice PLD has been observed during compatible as well as incompatible interactions of rice with Xoo as well as in response to wounding. However, the expression profile of PLD is different wherein there is an early rise in its expression during an incompatible interaction and the extent of its expression is much higher than during a compatible interaction. Also, the protein is found uniformly localized onto the plasma membrane during a susceptible (compatible) interaction whereas it is relocalized and gets clustered onto the plasma membrane at positions that are adjacent to the bacterial cells during 6 to 24 hr of an incompatible (or resistant) interaction.

The cell wall degrading enzymes (ClsA, CbsA and LipA) secreted by the Xoo T2SS induce rice defense responses [57]. Treatment with these purified enzymes induces cell death associated nuclear fragmentation in rice roots and root cap cells and HR associated browning reaction and lignin deposition in rice leaves. Infiltration of the soluble cell wall degradation products (elicitors) that are released by the action of these cell wall degrading enzymes on rice cell walls also results in visible HR and lignin deposition. The defense responses induced by these T2SS effectors impart resistance against further Xoo infection. Wild type Xoo is capable of suppressing these defense responses in a T3SS dependent manner. The ability to suppress these defense responses appears to be critical for Xoo to cause disease on rice as prior inoculation with a T3SS deficient mutant of Xoo immunizes rice against subsequent infection by the wild type strain of the pathogen. The defense responses that are induced by the T3SS deficient mutant of Xoo appear to be due to the activity of the T2SS as a $T2SS^{-}$ $T3SS^{-}$ double mutant of Xoo is very much compromised in the ability to induce these defense responses.

The concentration of the Xoo T2SS effectors appears to determine the kind of defense response that is induced in rice [57]. At a lower concentration (~100μg/ml), these effectors induce deposition of callose which fortifies plant cell walls without either visible HR or lignin deposition. At a higher concentration (>500 μg/ml), these effectors induce visible HR and lignin deposition. This suggests the possibility that the rice plant might be able to monitor the extent of damage to its cell wall, possibly by measuring the concentration of cell wall degradation products (that are released by the action of T2SS effectors) and accordingly calibrating its response. Higher amounts of damage may result in the initiation of a cell death pathway while lesser amounts of damage may result in the initiation of basal defense responses such as callose deposition.

As indicated above, lipopolysaccharides (LPS) have been shown to induce host defense responses in both plants and animals. The mechanism by which LPS is recognized by plant cells and the signal transduction pathways that are involved are not well characterized. Recently, Desaki *et al.* have shown that LPS (from several different bacteria) can induce defense responses such as programmed cell death, increased production of reactive oxygen species and increased expression of several defense genes in tissue cultured rice cells [137]. It is still to be determined whether LPS can induce such defense responses in whole plants and whether prior treatment of rice with purified LPS can induce resistance against subsequent Xoo infection.

Rice '*R*' Genes

There are more than 25 rice loci that are known to impart resistance against different Xoo strains [138]. These are the so called resistance or '*R*' genes. Some of these '*R*' genes provide resistance against a limited set of Xoo strains while others are effective against the majority of Xoo strains. A few of the rice '*R*' genes that provide resistance against Xoo have been cloned. The characteristics of these '*R*' genes are described in the following pages.

Xa1: *Xa1* encodes a cytoplasmic protein containing two nucleotide-binding domains (NBS) and leucine rich repeats (LRR) composed of six (93 aa each) almost perfect repeats [139]. Amongst the cloned rice '*R*' genes that confer resistance to Xoo, the domain architecture of *Xa1* is the most typical of proteins encoded by a number of other '*R*' genes from plants [128]. Expression of the *Xa1* gene is induced by both compatible and incompatible strains of Xoo and also by wounding [139]. It is hypothesized that the induced expression of *Xa1* might enhance the efficiency of its interaction with a hypothetical factor from Xoo which is either directly or indirectly recognized by the *Xa1* protein to induce a resistance response.

Xa5: The *xa5* gene is a recessive '*R*' gene [140]. It encodes the γ-subunit of transcription factor IIA (TFIIAγ; required by RNA polymerase II during transcription) and is located on chromosome 5 of rice. In the resistance allele of *xa5*, the amino acid valine (V; hydrophobic in nature) is present at the 39th position in the protein whereas in the susceptible allele it is replaced by a hydrophilic amino acid, glutamic acid (E), due to a substitution of two nucleotides. This substitution might significantly alter the structure of the *xa5* protein and may change its interaction with the acidic transactivation domain (AAD) of the AvrBs3 family of transcription activators that are secreted into rice by Xoo using the T3SS. A paralog of *Xa5* (TFIIAγ) is found to be present on chromosome 1 (TFIIAγ1) of rice and the expression of TFIIAγ1 is upregulated by an AvrBs3 type of transcriptional activator that is secreted into rice cells by some Xoo strains [83]. These results suggest that members of the AvrBs3 family of transcriptional activators interact with TFIIAγ of RNA Polymerase II to modulate rice transcription in a manner that it creates a favorable environment for the pathogen. In plants that are homozygous for the *xa5* resistance allele, it is presumed that this interaction between members of the AvrBs3 family of transcription activators and TFIIAγ of RNA Polymerase II is affected in such a manner that the in planta environment is not made favorable for growth of the pathogen.

Xa13: The *xa13* gene is another recessive '*R*' gene of rice. Therefore, like *xa5*, this gene can also confer resistance only when the resistance allele is present in a homozygous state. The gene for *xa13* has been cloned recently [141] and it is located on rice chromosome 8. The *xa13* gene consists of five exons which encode a protein showing homology with an early nodulin gene (*MtN3*) of *Medicago truncatula* (a legume plant) whose expression is inducible by Rhizobial strains. The recessive *xa13* containing rice lines differ from the dominant *Xa13* lines by the presence of small insertions, deletions or substitutions of nucleotide sequences in the promoter region of the gene. This leads to differences in the expression of this gene in such a manner that expression of the *OsMtN3* gene is highly induced upon pathogen inoculation of rice lines that contain the *Xa13* allele whereas the expression of the *OsMtN3* is not induced upon pathogen inoculation of rice lines that have the *xa13* allele in a homozygous condition [141]. Inactivation of *Xa13* through RNAi makes the plants resistant to Xoo infection indicating that induced expression of *Xa13* is required for pathogen growth and virulence [82]. As indicated earlier in this review, the upregulation in expression of *OsMtN3* is brought about by the activity of the PthXo1 protein which is secreted through the Xoo T3SS.

The *OsMtN3* gene is preferentially expressed in the parenchymatous cells surrounding the vascular elements of rice [141]. What is the role of *OsMtN3* in normal rice growth and development? The expression of *OsMtN3* is upregulated in panicles of rice plants containing either the *Xa13* or *xa13* alleles. Rice plants in which the expression of *Xa13* has been knocked down by RNAi are male sterile due to defects in pollen development [141]. Therefore it appears that *OsMtN3* is required for normal pollen development. However, it is not clear how *OsMtN3* promotes either pollen development or pathogen growth but it is possible that it might be involved in nutrient transfer to either the developing pollen grains or Xoo. It is interesting to note that the *xa13*/*xa13* rice lines do show near normal levels of pollen fertility. This suggests that the *xa13* mutation affects upregulation of the expression of *OsMtN3* by pathogen effectors without detrimental affects on expression of this gene during pollen development.

***Xa21*:** *Xa21* is a dominant 'R' gene. It is the first '*R*' gene to be cloned from rice and has been extensively characterized. The *Xa21* gene encodes a receptor like protein kinase and is located on chromosome 11 [142]. It imparts resistance against a broad spectrum of Xoo strains belonging to different races of the pathogen [142-144]. The protein has an interesting domain architecture comprising of a predicted extracellular region consisting of 24 aa long leucine rich repeats (LRR) that are present

23 times and a transmembrane region followed by a serine-threonine kinase like cytoplasmic region. *Xa21* belongs to a multigene family, all members of which are present at the same locus. The functions of most of the other members of this gene family are not known. Gene expression analysis has revealed that the *Xa21* gene is constitutively expressed and that expression is not dependent on the age of the plant, the presence of the pathogen (Xoo) or wounding [145]. However, the resistance response triggered by *Xa21* is developmentally regulated wherein the *Xa21* containing line is susceptible to Xoo infection in the seedling stage with full resistance being observed only in adult plants [145]. This regulation is post-transcriptional but the exact mechanistic basis for this phenomenon has not yet been determined. The cellular level of the *Xa21* protein is regulated by proteolytic cleavage at/or near the Ser 686/Thr688/ Ser689 residues [146]. The molecule involved in proteolytic cleavage of Xa21 has not yet been identified but has been hypothesized to be developmentally regulated. The *Xa21* protein demonstrates autophosphorylation activity. The autophosphorylation at the Ser 686/Thr688/Ser689 residues enhances the stability of *Xa21* and substitution of these residues with alanine leads to destabilization of the protein. Transgenic rice plants expressing such mutated proteins demonstrate reduced levels of resistance against Xoo. In an effort to identify rice proteins associated with Xa21 mediated resistance, a protein named XB3 (Xa21 binding protein 3) has been identified [147]. The XB3 is an E3 ubiquitin ligase containing RING finger motif and ankyrin repeat. RNAi mediated down regulation of *Xb3* leads to reduction of *Xa21* concentration, probably by destabilizing its transcript. This in turn reduces the effectiveness of the *Xa21* resistance gene.

***Xa26*:** *Xa26* is a dominant '*R*' gene which, like *Xa21*, is also located on chromosome 11. The domain architecture of the Xa26 protein is similar to that of Xa21. It consists of 24aa long LRR motifs that are present 26 times in an N-terminal extracellular domain followed by a transmembrane region and a putative cytoplasmic protein kinase domain at the C-terminal [148]. The *Xa26* gene is constitutively expressed and there is no difference in its expression due to inoculation with either different Xoo strains or mock inoculation with water. *Xa26* mediated resistance is not developmentally regulated and is effective in the early juvenile to the adult stages. Based upon the map based location and further molecular characterization it seems that *Xa26* is identical to the previously described *Xa3* and *Xa22(t)* genes which confer resistance against Xoo. The *Xa3*/*Xa26* gene is a member of a multigene family. The *MRKa*, *MRKc* and *MRKd* (a pseudo gene) are other members of this gene family in rice cultivar Minghui 63 [149]. Expression of the *Xa3*/*Xa26*, *MRKa* and *MRKc* genes occurs in vascular systems of different tissues of rice. Constitutive expression of *MRKa* under the control of a strong promoter has been shown to impart partial resistance against Xoo infection. However, transgenic rice plants expressing the gene under the control of its native promoter do not exhibit such resistance. The genetic background of the rice cultivar and the amount of *Xa3/ Xa26* transcripts are postulated to govern its functionality [150]. The gene is found to be more effective in japonica rice cultivars than in indica cultivars.

Xa27: *Xa27* is another dominant rice '*R*' gene that has been cloned recently [78]. It is located on chromosome 6 and is found to be intron less. The predicted protein product of this gene does not demonstrate homology with any other proteins in the databases. The resistance allele of the gene differs from the susceptible allele only in the promoter region and both encode identical proteins [78]. The difference in the promoter region leads to a difference in expression of this gene wherein only the resistance allele is expressed and that too only upon inoculation with the incompatible Xoo strains containing the corresponding *avrXa27* gene. The *avrXa27* gene of Xoo has also been cloned and it has been shown to encode a T3SS secreted protein that is a member of the AvrBs3 family of transcriptional activators [78]. The *Xa27* resistance allele is considered to be a case of molecular mimicry wherein the promoter of the gene for a rice anti-microbial protein has evolved such that it is now a binding site for a transcriptional activator (AvrXa27) that is secreted into rice cells by Xoo. In plants having the resistance allele, the AvrXa27 protein binds to the promoter of the *Xa27* gene and upregulates its expression leading to resistance. In plants having the susceptible allele, the AvrXa27 protein does not bind to the promoter of this gene and hence its expression is not upregulated upon Xoo infection. This results in susceptibility.

Rice Defense Signaling Pathways and Resistance to Xoo

The rice genome contains more than 100 WRKY transcription factors and one of them, namely *OsWRKY71,* has been found to be upregulated following either pathogen attack or wounding as well as by inducers of plant defense responses such as salicylic acid (SA) and jasmonic acid (JA) [151]. Overexpression of *OsWRKY71* induces resistance in rice against Xoo infection and leads to constitutive expression of *OsNPR1*, a key player in plant defense signaling pathways [151]. Overexpression of the Arabidopsis *NPR1* gene in rice imparts resistance against Xoo infection [152]. Over expression of *OsNPR1* also leads to enhanced resistance against Xoo infection but makes

the plants more susceptible to herbivore attack [153]. The OsNPR1 protein is localized in the cytoplasm but is relocated into the nucleous following changes in redox potential. Overexpression of an OsNPR1 mutant, in which certain conserved cysteine residues have been replaced, leads to constitutive localization in the nucleous and provides resistance against Xoo infection without any enhanced susceptibility to herbivore attack. The plant hormone Indole-3-acetic acid (IAA) has also been implicated in rice defense responses. Prevention of IAA accumulation by overexpression of *GH3-8* (a rice IAA amido synthetase gene) induces resistance in rice against Xoo infection. This induced resistance seems to be independent of JA or SA defense signaling pathways [154]. Genome wide screens, using microarray and proteomics tools, have also been initiated to characterize rice resistance responses against Xoo infection [155-157]. These are expected to provide new insights into the signaling pathways and transcription factors that are operative during rice defense responses that are induced following Xoo infection.

Lesion Mimic Mutants of Rice

Several lesion mimic mutants that undergo spontaneous cell death (that is similar to the HR), even in the absence of the pathogen have been isolated in rice. Some of the mutations that cause lesion mimic phenotype are dominant in nature suggesting that they might be positively activating defense responses. The recessive mutations that cause a lesion mimic phenotype might be in genes which are negative regulators of defense responses. A number of rice lesion mimic mutants have been found to be resistant to infection by both Xoo and the fungal pathogen, *Magnaporthe grisea* [158-160]. The genes for several pathogenesis related proteins, which are found to be upregulated following pathogen infection in a number of plants, are over expressed in these mutants even in the absence of infection. Further work has to be done to elucidate the molecular basis for the lesion mimic phenotype and associated disease resistance in rice.

Developing Disease Resistant Rice Varieties by Molecular Breeding

Chemical control for BB is not very effective [161]. Therefore, the development of resistant host varieties represents the best option for disease management [162]. As indicated above, more than 25 different 'R' genes have been identified that confer resistance against different races of Xoo. Although only six of these 'R' genes have been cloned, several others have been tagged with closely lined molecular markers that can be used in marker assisted selection. A number of rice varieties which contain single 'R' genes like *Xa4* have been developed using conventional plant breeding [162]. However, Xoo has been known to overcome rice varieties with single 'R' genes [163]. Therefore, the current strategy is to develop, using molecular marker assisted selection, rice varieties that contain multiple 'R' genes as this might be expected to lead to a more durable resistance.

DNA fingerprinting and pathotype analysis have indicated that there is a significant amount of diversity within populations of Xoo in India and other rice growing countries [164-170]. Near-isogenic rice lines, each of which contains a different 'R' gene in the genetic background of IR24 rice variety, were inoculated with Indian strains of Xoo and the single resistance genes *Xa21*, *xa13* and *xa5* were found to provide moderate to strong levels of resistance against these strains [168, 171, 172] (Yashitola Jamir and R. V. Sonti, unpublished results). PCR based molecular markers that are tightly linked to each of these three resistance genes are available [173-175] and have been used to pyramid them into the genetic background of different commercially important rice varieties [172, 176-180]. The three-gene pyramids containing *Xa21*, *xa13* and *xa5* have been tested against multiple Indian isolates of Xoo and were found to exhibit high levels of resistance. The pyramid lines containing these 'R' genes in the genetic backgrounds of rice varieties Pusa-Basmati-1 and Samba Mahsuri have undergone national field trials under the All India Coordinated Rice Improvement Project of the Indian Council of Agricultural Research and released for commercial cultivation.

Summary and Future Prospects

The studies reviewed here indicate that the Xoo pathogen uses a diverse array of virulence factors and that the rice plant defends itself against Xoo attack using a variety of resistance genes. One of the striking findings that has emerged is that Xoo proteins are targeted into rice cells to upregulate expression of host susceptibility factors and that some of the resistance genes/alleles are subtle mutations in the promoters of host susceptibility genes or transcriptional apparatus which prevent the commandeering of rice genes by the pathogen. The subtle nature of these genetic changes indicates that intense selective pressure is operating on both interacting partners and that on one side this leads to novel virulence factors and on the other side towards newer resistance genes. Closely linked molecular markers have been developed for several rice resistance genes. These markers have been used, in molecular marker assisted selection, to pyramid multiple BB resistance genes into commercially important rice cultivars. Some of these newly developed BB resistant lines have been released for commercial cultivation. Studies on the application of microarray and proteomic tools for understanding the

xanthomonas-rice interaction have been initiated. In the coming years, these are expected to provide newer insights into the strategies employed for attack and defense in this very interesting and agriculturally important pathosystem.

Acknowledgements

Research in the RVS lab is supported, in part, by grants from the Department of Biotechnology, Government of India and the AMAAS Project of the Indian Council of Agricultural Research, New Delhi.

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