Bacterial leaf blight of rice: New insights from molecular genetics

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The bacterial pathogen *Xanthomonas oryzae* pathovar *oryzae* causes a serious disease of rice. Over the last decade, considerable progress has been made in the application of the techniques of genetics and molecular biology towards understanding the rice–*Xanthomonas* interaction. Several interesting pathogen genes for virulence and avirulence have been cloned. DNA fingerprinting studies have provided information about the genetic diversity within populations of this pathogen. A number of rice genes that confer resistance against *X. oryzae* pv. *oryzae* have been tagged with closely linked molecular markers. Rice breeding lines with multiple resistance genes have been obtained by using these markers. The sequence of one of these genes suggests that it encodes a putative receptor kinase which recognizes an elicitor produced by the pathogen and initiates a signal transduction cascade that ultimately results in resistance.

Xanthomonas oryzae pathovar *oryzae* causes bacterial leaf blight, a serious disease of rice (Box 1). The causal organism is a gram-negative bacterium belonging to family Pseudomonadaceae[1]. Almost all known members of the genus *Xanthomonas* are plant pathogens and cause a number of economically important diseases. Athough the genus as a whole has a broad host range, individual members are specialized for causing disease on related species of plants. *X. oryzae* pv. *oryzae* causes disease on cultivated rice and its wild relatives.

Leaf blight symptoms are characterized by yellowing and drying which begin at the tips of rice leaves and spread down one or both sides of the leaf or through the mid-vein (Figure 1). The pathogen gains entry into the plant either through wounds or natural openings called hydathodes that are concentrated at the edges of rice leaves[2]. The bacterium multiplies inside the xylem vessels and travels through them to more distant parts of the leaf or in some cases to other parts of the plant. The bacterium exits from the leaf in the form of ooze drops that collect on the leaf surface. Wind and rain contribute to dispersal of the pathogen, by splashing the ooze onto uninfected plants[3]. The pathogen might survive when rice is not cutivated either in dry plant parts left over from the previous harvest, in seeds or on wild relatives of rice[4]. Several reviews of the pathology and epidemiology of *X. oryzae* pv. *oryzae* are available and can be referred to for details[5,6].

Bacterial leaf blight affects the rice crop in all major rice-growing countries of Asia and has also been reported in Africa, Australia and the Americas[7]. In India it is a serious problem during the south-west monsoon season. The high wind and rain that are experienced during this time of the year are believed to aid in rapid dispersal of the pathogen. In severe infections, yield losses can range from 20 to 40%. The application of molecular genetic techniques is providing an increased understanding of the basic biology of the rice–*X. oryzae* pv. *oryzae* interaction and opening up new possibilities for reducing yield losses. This review is a summary of recent advances made in this area.

Figure 1. Disease symptoms of *Xanthomonas oryzae* pv. *oryzae* infection on rice. ***a***, A healthy rice leaf. ***b***, Leaf blight symptoms that are caused by *Xanthomonas oryzae* pv. *oryzae*. The disease lesion is advancing down the mid-rib.

Development of methodology

Molecular genetic studies on this bacterium were initiated about a decade ago[8]. Since then, several broad host range plasmids that will replicate in *X. oryzae* pv. *oryzae* have been identified and genomic libraries constructed[9–13]. Protocols have been standardized for transfer of plasmids into this bacterium either by conjugation with donor *Escherichia coli* strains or by electroporation[14,15]. Two different restriction–modification (r–m) systems are present in certain Philippine strains of *X. oryzae* pv. *oryzae*[16]. A strong restriction barrier has been found in an Indian *X. oryzae* pv. *oryzae* strain that lacks both of the previously identified systems[17]. It is therefore likely that other r–m systems are present in this bacterium. Neutralization of the restriction barrier either by mutation or by cloning the appropriate modification enzyme/s will greatly facilitate molecular genetic studies on this bacterium. The observation that mutagenesis using mini-transposon 5 derivatives[18,19] can be performed in *X. oryzae* pv. *oryzae*[20] indicates that transposon tagging methods can be used to clone and characterize interesting genes. Insertion element sequences that are native to the genome of *X. oryzae* pv. *oryzae* have been identified and their ability to transpose has been demonstrated[21,22]. These insertion elements have been used as probes for DNA fingerprinting of *X. oryzae* pv. *oryzae* strains (see later section for details).

Extracellular polysaccharide-deficient mutants

Like other xanthomonads, *X. oryzae* pv. *oryzae* produces copious amounts of extracellular polysaccharide[23] (EPS). Spontaneous EPS and virulence-deficient mutants have been described[24,25]. These mutants accumulate only in prolonged stationary phase cultures and not in exponentially growing cultures, suggesting that starvation may contribute either to the formation or selection of these mutants[26]. Several stationary phase selected EPS and virulence-deficient mutants are hypersensitive to hydrogen peroxide, suggesting that functions other than those directly involved in EPS synthesis may be mutated in these strains. A gene that encodes a positive regulator of EPS biosynthesis has been cloned[12]. Mutations in this gene affect symptom expression but do not impair growth in rice. Loss of an endogenous plasmid has also been associated with EPS and virulence deficiency[27]. The virulence deficiency associated with these various mutants suggests that EPS is an important virulence factor. However, in all of the above it is not possible to state categorically that the virulence defect is solely due to the EPS deficiency as other functions may be affected either by plasmid loss or by mutations in regulatory genes.

Genetics of pigment synthesis

Xanthomonadin is a yellow, membrane bound brominated aryl polyene pigment that is a characteristic feature of the members of the genus *Xanthomonas*[1,28] (Box 2). Characterization of pigment-deficient mutants suggests that xanthomonadin is not essential for virulence of *X. oryzae* pv. *oryzae*[29,30] but may provide protection against photo-biological damage[31]. A heterologous probe from *Xanthomonas campestris* pv. *campestris*[32], which causes disease on plants like cabbage and mustard, has been used to clone a cluster of *X. oryzae* pv. *oryzae* genes involved in xanthomonadin biosynthesis[33]. Reporter gene fusions are being made to promoters of pigment genes to assess their regulation; specifically to determine whether they are expressed during growth within the rice plant.

Hypersensitive response and pathogenicity mutants

The interaction between a particular strain of a plant pathogen and its host plant can result in either resistance or susceptibility. In a resistance response, the presence of the pathogen is detected in a timely manner by the host and a defense response is mounted which limits spread of the pathogen. This response is characterized by hypersensitivity to the pathogen and results in death of host cells in the infected area along with those of the pathogen[34]. If the pathogen is able to evade the host recognition mechanism (see next section for details) it can multiply and cause disease. Infection of a non-host plant can also lead to induction of a hypersensitive response. The hypersensitive response of tomato is easily visualized as rapid necrosis of infected portions of the leaf. Mutants of *X. oryzae* pv. *oryzae* have been isolated that do not induce a hypersensitive response in the non-host tomato[35]. These mutants are also severely deficient for virulence on rice and have been called *hrp* (hypersensitive response and pathogenicity) mutants.

Hrp^- mutants were reported earlier in several plant pathogenic bacteria and the affected genes have been characterized[36]. They are present in gene clusters spanning 24–40 kb. These genes are homologous[37] to previously characterized protein transport systems in several animal pathogenic bacteria[38,39] which are dedicated specifically for export of bacterial proteins (virulence factors) into eukaryotic host cells.

A *hrp* gene cluster has been isolated from *X. oryzae* pv. *oryzae* and partially characterized[15]. An interesting observation is that co-inoculation with a Hrp^+ strain will rescue, in plants, the virulence deficiency of a *hrp* mutant. This is consistent with the possibility that the *hrp* genes of *X. oryzae* pv. *oryzae* are encoding a protein export system. However, very little information is avail-

Box 1. Rice diseases of special relevance to India

Besides bacterial leaf blight, several other serious rice diseases like blast, sheath blight and tungro occur in India.

Rice blast caused by the fungus, *Pyricularia grisea*, is one of the most destructive diseases of rice. The pathogen affects rice and several other grasses. Disease lesions occur on all aerial parts of the plant, except the leaf sheath. A particularly destructive phase of the disease, called panicle blast, is associated with infection of the rice panicle (inflorescence). Cultivation of resistant varieties, application of fungicides and implementation of appropriate cultural practices are being employed for disease control.

Sheath blight is another serious disease of rice. The causal organism, *Rhizoctonia solani*, is capable of infecting several grass species as well as other plants. Disease lesions are initiated on the leaf sheath, and under favourable environmental conditions, spread rapidly to other aerial parts of the plant. Rice cultivars that are resistant to this disease have not yet been obtained and the application of fungicides is the most effective means of disease control.

Tungro is the most serious viral disease of rice. It is caused by infection with rice tungro bacilliform virus (RTBV) and rice tungro spherical virus (RTSV). The RTBV genome is a circular double-stranded DNA molecule while the RTSV genome is a single-stranded RNA molecule. The viruses are transmitted through the green leaf hopper insect vector, *Nephotettix virescens*. Symptoms are characterized by stunting of plants and a yellow to yellow–orange discoloration of leaves. Disease control measures are aimed at controlling the insect vector and the cultivation of resistant varieties, crop sanitation and crop rotation.

Besides the above-mentioned diseases, the rice plant is subjected to more than 50 other diseases caused by a number of viral, bacterial and fungal pathogens. Fortunately, at present, many of these diseases are not of a serious nature. However, future changes in agricultural practices might make some of these diseases much more serious than they are today. (Source: Webster, R. K. and Gunnell, P. S., *Compendium of Rice Diseases*, The American Phytopathological Society, USA, 1992.)

able about the candidate proteins that might be exported through such a transport system. A Hrp$^-$ phenotype is also caused by mutations in a gene that appears to encode a positive regulator of other *hrp* genes[40,41].

Several Hrp$^+$, virulence-deficient mutants of *X. oryzae* pv. *oryzae* have also been identified. Unlike the Hrp$^-$ strains, the virulence deficiency of these mutants is not complemented by co-inoculation with the wild type strain, suggesting that the mutants are deficient in functions that act within individual bacterial cells[42]. These mutants are EPS$^+$ and do not appear to have any general growth or nutritional deficiencies. Cloning and characterization of the affected genes is likely to provide more information about their role in virulence.

Avirulence genes

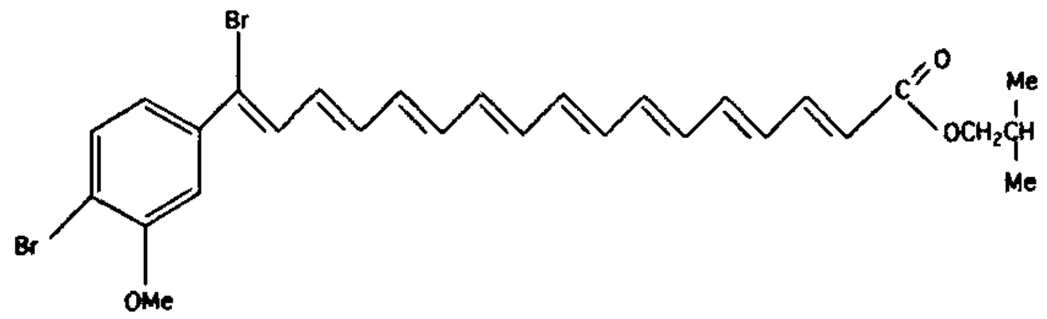
On the basis of the genetic analysis of interaction between a pathogenic fungus and flax plants, it was postulated that resistance is determined by a gene for gene interaction between the plant and the pathogen[43]. According to this hypothesis, host plant resistance is triggered by the specific interaction between a resistance gene product and an elicitor produced by the pathogen. The pathogen gene that controls elicitor production is called an avirulence gene because its activity makes the microbe avirulent. It was suggested that the pathogen contains a number of avirulence genes and that each of these avirulence genes has cognate resistance genes in the host. This hypothesis has been largely validated by the cloning of several avirulence genes from phytopathogens and disease resistance genes from plants[44,45].

A family of avirulence genes, that had been previously described in several other xanthomonads, has also been identified in *X. oryzae* pv. *oryzae*. Two of the members have been characterized extensively[10] and each of them is recognized by a specific resistance gene in the rice plant. Although 7–14 members of this family are present in *X. oryzae* pv. *oryzae* strains[10,44], all of these genes may not be functional. The mode of action of these avirulence genes in eliciting host plant resistance is unknown. The elicitor may either be the avirulence gene product itself or a result of its activity[44]. Avirulence gene activity is dependent on the presence of functional *hrp* genes[44]. One possible model that has emerged is that the avirulence gene products are directly transported into plant cell nuclei through the *hrp* system. Consistent with this possibility, an avirulence gene product of the cotton pathogen, *Xanthomonas campestris* pv. *malvacearum*, has been found to contain signals that will target it to plant nuclei[46].

The avirulence genes pose a paradox. Why should the pathogen carry genes that allow the host to recognize it? The answer may be that the avirulence gene products function as virulence factors and thereby confer a selective advantage to the pathogen. It has indeed been found that at least some members of this gene family are required for optimal virulence[44,47]. During evolution, plants might have elaborated resistance genes that recognize these virulence functions as a cue for mounting

Box 2. Structure of xanthomonadin I

Xanthomonadins are brominated, aryl polyene pigments that are characteristic of the genus *Xanthomonas*. Very little information is available about xanthomonadin biosynthesis except for the suggestion that the aromatic ring may be derived from the shikimate pathway and that the polyene chain may be derived from the polyketide pathway. No information is available about the steps involved in bromination.



a defense response. The virulence genes are thus converted into avirulence genes. A game of evolutionary hide and seek might have been initiated with the pathogen mutating its avirulence genes to evade recognition by the host plant, which in turn evolves new resistance genes to detect the modified forms of the pathogen.

Host resistance genes

At least 19 rice loci that condition resistance against various strains of *X. oryzae* pv. *oryzae* have been identified[48,49]. A majority of the resistance genes are dominant in nature while a few like *xa-5* and *xa-13* are recessive[50]. Some resistance genes are active only at particular stages of plant development[51]. Nine different resistance genes have been tagged by closely linked molecular markers. These genes are *Xa-3*, *Xa-4*, *xa-5*, *Xa-10* (ref. 52), *xa-13* (ref. 53), *Xa-1* (ref. 54), *Xa-7*, *Xa-11* (ref. 55) and *Xa-21* (ref. 56). The molecular markers can be used as indirect indicators of the presence of a resistance gene, i.e. in marker-aided selection. They are of advantage to plant breeders as they facilitate the creation of rice lines carrying multiple resistance genes by obviating the necessity to conduct phenotypic assays for the presence of each resistance gene. With the aid of these markers, 16 rice lines that are homozygous for particular pairs of resistance genes have been obtained[52,55]. Rice lines that are homozygous for four different resistance genes have also been reported[57]. This is significant because incorporation of multiple resistance genes into rice cultivars, a process called gene pyramiding, can be expected to result in more broad based and durable resistance. The rice lines that carry multiple resistance genes exhibit a broader spectrum and higher level of resistance than the single gene containing parental lines[57]. Several quantitative trait loci (QTL) that provide resistance against *X. oryzae* pv. *oryzae* have also been tentatively localized, using molecular markers to certain regions of rice chromosomes[55,58].

The *Xa-21* disease resistance gene has been isolated by map-based cloning methods[59]. This gene is derived from *Oryza longistaminata*, a wild relative of *Oryza sativa* (the cultivated rice). It was initially crossed into the cultivated rice background[60,61], and tagged with closely linked molecular markers[62,63]. *Xa-21* belongs to a gene family whose other members also cluster to the same genomic region[64]. Sequence analysis indicates that the *Xa-21* gene encodes a putative receptor kinase with an extracellular domain that contains several leucine-rich repeats. Similar repeats in other proteins have been implicated in protein–protein interactions. The extracellular domain of *Xa-21* might interact with an elicitor (possibly a peptide) that is released by the pathogen. This interaction might activate the kinase domain of the protein which can then initiate a signal transduction cascade that leads to resistance. Sequence comparison indicates that the *Xa-21* gene is homologous to several other disease resistance genes that have been cloned from plants[65,66]. Another interesting observation is that the *Xa-21* gene confers resistance against a majority of *X. oryzae* pv. *oryzae* strains that were collected from a number of countries[67]. This is unusual for a single resistance gene and suggests that it might either recognize multiple pathogen avirulence genes or a single avirulence gene that is widely distributed in the pathogen population.

A yeast artificial chromosome (YAC) clone that carries the *Xa-1* disease resistance gene has been identified[68] and recently, the isolation of this gene has also been reported[69]. The deduced amino acid sequence of the *Xa-1* gene indicates that it has leucine-rich repeats and is in this respect similar to *Xa-21*. This is consistent with the emerging theme that plant resistance genes contain leucine-rich repeats that are involved either in recognizing elicitors produced by the pathogen or in interaction with other components of the resistance signaling pathway. Expression of the *Xa-1* gene is reported to be undetectable in uninfected leaves but is induced by inoculation with the pathogen[69].

Induction of the rice resistance response against *X. oryzae* pv. *oryzae* has been shown to be associated with accumulation of a cationic peroxidase in the xylem vessels[70,71] as well as increased synthesis and deposition of lignin[72]. The cationic peroxidase appears to be synthesized in adjacent parenchyma cells and transported to the xylem vessels. A redistribution of membrane phospholipase D in close juxtaposition to points of contact with *X. oryzae* pv. *oryzae* has also been reported[73]. The exact contribution of each of these changes towards the development of resistance needs to be determined along with an understanding of the other physiological and biochemical changes that occur during a resistance response.

Genetic engineering

As indicated above, the *Xa-21* gene provides protection against a number of *X. oryzae* pv. *oryzae* strains. This raises the possibility that bacterial leaf blight resistance can be introduced directly into elite rice cultivars by genetic transformation with the *Xa-21* gene. This was shown to be possible by the introduction of *Xa-21* into the improved rice varieties IR64 and IR72 (ref. 74). An alternative strategy is to express peptides or proteins with bactericidal activity in rice. Initial results suggest that rice plants expressing cecropin B (which is a 36 amino acid basic polypeptide isolated from a giant silkworm) exhibit enhanced resistance against *X. oryzae* pv. *oryzae*[75].

DNA fingerprinting methods

Traditionally population diversity has been determined by examining differences in the ability of individual strains to cause disease on rice cultivars that carry different resistance genes. This method is time-consuming and the results are affected by environmental factors. DNA fingerprinting methodology allows numerous loci to be screened under controlled conditions in the laboratory. Several multi-locus probes for RFLP analysis have been developed[21,22] and used to determine the genetic diversity in *X. oryzae* pv. *oryzae* populations[22,76–79]. These probes include endogenous insertion sequences and an avirulence gene. A microsatellite oligonucleotide probe as well as a human minisatellite probe have also been shown to detect substantial genetic diversity in *X. oryzae* pv. *oryzae*[80]. Restriction digestion of genomic DNA with the enzyme *PstI* and visualization by ethidium bromide staining after agarose gel electrophoresis has been applied to detect pathogen variability[81]. However, the large number of fragments that are generated by this method makes it difficult to score the identity of individual fragments with certainty.

PCR-based methods have been developed for assessing genetic diversity of *X. oryzae* pv. *oryzae*[82,83]. These methods are more efficient and are useful for rapid evaluation of a large number of samples. The PCR primers that were initially used for DNA fingerprinting of *X. oryzae* pv. *oryzae*[82] were based on the consensus sequences of small, intergenic repetitive elements that are present in many bacterial genomes[84–86]. Subsequently primers that require shorter amplification times were developed[83]. These are based on the end sequences of *X. oryzae* pv. *oryzae* insertion elements and were designed to amplify genomic sequences that lie between adjacent insertions of these elements. The groupings of strains obtained by PCR analysis are similar to those obtained by RFLP analysis[82,83].

An interesting aspect of pathogen population structure that has emerged from the DNA fingerprinting studies is the observation that closely related isolates of the pathogen can be present in widely separated geographic locations. This was initially suggested by Adhikari *et al.*[78] who surveyed strains from many locations in Asia and observed that certain groups contained strains from different countries. George *et al.*[83] reported that one closely related group of *X. oryzae* pv. *oryzae* constitutes 98% of strains in a collection of Indonesian isolates and 86% of strains in a collection of Philippine isolates. This distribution is observed in spite of the presence of ocean barriers that separate these two countries. Yashitola *et al.*[79] observed that another closely related group of strains constitutes 82/90 strains in a collection of Indian isolates of *X. oryzae* pv. *oryzae*. These strains were collected from 23 locations in India that are separated from each other by several hundred to several thousand kilometres. Since multi-locus probes were used in these studies, it is unlikely that the related strains arose independently at various locations and suggests that pathogen migration has occurred. One way in which the pathogen can migrate across such large distances is through infected seed.

DNA fingerprinting studies on Indian *X. oryzae* pv. *oryzae* strains are currently being performed by several groups[79,80,87]. These studies have not yet led to a uniform picture of the population structure of this pathogen in India. This may be due to the different probes that are being used by the various groups.

Future prospects

The research reviewed indicates that considerable progress has been made during the last decade in our understanding of the rice–*Xanthomonas* interaction. Future research can be expected to provide a clearer picture of pathogen population structure and modes of migration; as well as increase our understanding of the functions of virulence and avirulence genes and the role of *hrp* genes in the infection process. The characterization of rice resistance genes and intermediate steps in the resistance process will shed light on the mechanistic basis of host plant resistance. The application of molecular markers will facilitate studies of quantitative trait loci that confer resistance against *X. oryzae* pv. *oryzae*. From a practical perspective, marker-aided selection is likely to be increasingly used in developing rice cultivars that have favourable combinations of disease resistance genes. Bacterial leaf blight resistance will be introduced into elite rice cultivars by direct transformation using rice or non-rice genes that confer resistance against *X. oryzae* pv. *oryzae*.

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