Review

Recent progress and understanding of the molecular mechanisms of the rice–*Magnaporthe oryzae* interaction

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SUMMARY

Rice blast, caused by the fungal pathogen *Magnaporthe oryzae*, is the most devastating disease of rice and severely affects crop stability and sustainability worldwide. This disease has advanced to become one of the premier model fungal pathosystems for host–pathogen interactions because of the depth of comprehensive studies in both species using modern genetic, genomic, proteomic and bioinformatic approaches. Many fungal genes involved in pathogenicity and rice genes involved in effector recognition and defence responses have been identified over the past decade. Specifically, the cloning of a total of nine avirulence (Avr) genes in *M. oryzae*, 13 rice resistance (R) genes and two rice blast quantitative trait loci (QTLs) has provided new insights into the molecular basis of fungal and plant interactions. In this article, we consider the new findings on the structure and function of the recently cloned R and Avr genes, and provide perspectives for future research directions towards a better understanding of the molecular underpinnings of the rice–*M. oryzae* interaction.

INTRODUCTION

Rice is one of the most important food crops, feeding more than 50% of the world's population. Grain yield has been doubled since the 1960s with the help of the widespread cultivation of semi-dwarf and hybrid rice cultivars. However, a production bottleneck has developed as a result of a combination of factors, including limited germplasm, and biotic and abiotic stresses, with yield losses caused by disease averaging upwards of 30% (Skamnioti and Gurr, 2009). Of particular importance, rice blast has re-emerged as a major factor influencing stable rice production

and food security in many rice-growing countries in Asia and Africa. For example, in an average year, 50% of rice yield is lost in eastern Indian upland regions alone (Khush and Jena, 2009; Variar, 2007). Increases in temperature and average rainfall in this region have only exasperated the disease, as has been witnessed by increases in disease incidence and epidemics over the past few years, a situation that is only anticipated to worsen as global warming continues. In China, a large increase in the planted acreage of super-hybrid rice has prompted a corresponding increase in blast, presumably as a result of the relatively narrow genetic base of hybrid rice and the increased use of nitrogen fertilizer. About 20% of the hybrid rice fields in China were reported to have severe seedling and neck blast in 2006 in a report from the Ministry of Agriculture of China (<http://www.agri.gov.cn/xxgktjxx/>). In South-East Asia, there has been an increased severity of blast in Indonesia, Vietnam and the Philippines. This suggests that an erosion of resistance caused by pathogen evolution or a relaxing of screening efforts in breeding programmes, or both, are the main reasons for the loss of rice blast resistance. Blast is a growing concern in Africa because of the rapid increase in rice cultivation acreage and intensity to meet an annual 6% increase in consumption. Currently, blast occurs in the major rice production areas in eastern Africa, including Burundi, Rwanda and Tanzania. Therefore, there is an urgent need to develop novel strategies to breed new rice cultivars that confer high and stable resistance to this important rice disease in order to ensure food security in these developing countries.

DISTRIBUTION OF BLAST RESISTANCE (R) GENES AND QUANTITATIVE TRAIT LOCI (QTLs) IN THE RICE GENOME

The use of host resistance has been proven to be the most effective and economical method to control rice blast. Rice blast resistance is generally classified into two main types: complete (true) resistance and partial resistance (field resistance) (Ezuka, 1972; Parlevliet, 1979). Complete resistance is race-specific and

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Fig. 1 Distribution of the identified rice blast complete resistance genes and quantitative trait loci (QTLs). The main data were collected from the China Rice Data Centre (<http://www.ricedata.cn/>) and the paper by Yang *et al.* (2009). The mapped resistance genes and QTLs are located on the right side of the chromosomes. DNA markers are located on the left side of the chromosomes. Detailed information on the cloned genes is listed in Table 1.

controlled by a single dominant or recessive R gene that can be recognized by a cognate avirulence (Avr) gene in the pathogen (Skamnioti and Gurr, 2009). By contrast, partial resistance is non-race-specific and controlled by QTLs. It is not yet known whether there is a cognate Avr gene in the pathogen for a QTL in the host. To date, over 70 R genes have been identified, distributed among all chromosomes, except chromosome 3 (Yang *et al.*, 2009; Fig. 1). Interestingly, many R genes are clustered, especially on chromosomes 6, 11 and 12. Although many blast QTLs have been roughly mapped, the genomic positions of only nine QTLs have been defined by molecular markers (Fig. 1).

UNIQUE FEATURES OF R GENES

Thirteen complete R genes have been cloned in the last decade, as summarized in Table 1. Interestingly, except for *Pi-d2*, all R genes encode nucleotide-binding site and leucine-rich repeat (NBS-LRR)-encoding proteins, one of the largest families of plant R genes (Liu *et al.*, 2007a). The phylogenetic relationship of the 12 cloned NBS-LRR R proteins was analysed (Fig. 2). As expected, *Pi2*, *Pi9* and *Piz-t* are clustered into the same clade. However, the allelic genes *Pikm* and *Pikh*, at the bottom of

chromosome 11, are located in different clades relatively far apart. Two unique features can be observed in these R genes compared with those in other plant species. First, the *Pi2/9* locus contains a complex cluster of NBS-LRR genes with different specificities. The three cloned R genes, i.e. *Pi9*, *Pi2* and *Piz-t*, are all located in a 100-kb region on chromosome 6, although *Pi9* originates from the wild species *O. minuta* and the other two are from local cultivars. There are only eight amino acid differences between *Pi2* and *Piz-t* (Zhou *et al.*, 2006). Additional R genes have also been mapped in the region, i.e. *Pi40(t)*, *Pigm*, *Pi26* and *Piz*. *Pi40(t)* was introgressed from wild species *O. australiensis*, with the other three identified from local cultivars (Deng *et al.*, 2009; Jeung *et al.*, 2007; Wu *et al.*, 2005). Interestingly, all confer broad-spectrum resistance to different sets of blast strains collected from different countries. The large number of closely related NBS-LRR genes clustered in the region may contribute to the generation of new R alleles through recombination and uneven crossing over. It is expected that more R specificities will be identified at the locus from different local cultivars or wild rice accessions. The second unique feature is that two NBS-LRR genes are required for *Pikm*- and *Pi5*-mediated resistance (Ashikawa *et al.*, 2008; Lee *et al.*, 2009).

Table 1 The characteristics of the cloned blast resistance genes and quantitative trait loci (QTLs) in rice.

R gene	Encoding protein	Cognate Avr gene	Reference
<i>Pib</i>	NBS-LRR	NA	Wang <i>et al.</i> , 1999
<i>Pita</i>	NBS-LRR	Yes	Bryan <i>et al.</i> , 2000
<i>Pi9</i>	NBS-LRR	NA	Qu <i>et al.</i> , 2006
<i>Pi2</i>	NBS-LRR	NA	Zhou <i>et al.</i> , 2006
<i>Piz-t</i>	NBS-LRR	Yes	Zhou <i>et al.</i> , 2006
<i>Pi-d2</i>	Receptor kinase	NA	Chen <i>et al.</i> , 2006
<i>Pi36</i>	NBS-LRR	NA	Liu <i>et al.</i> , 2007b
<i>Pi37</i>	NBS-LRR	NA	Lin <i>et al.</i> , 2007
<i>Pikm</i>	NBS-LRR	Yes	Ashikawa <i>et al.</i> , 2008
<i>Pit</i>	NBS-LRR	NA	Hayashi and Yoshida, 2009
<i>Pi5</i>	NBS-LRR	NA	Lee <i>et al.</i> , 2009
<i>Pid3</i>	NBS-LRR	NA	Shang <i>et al.</i> , 2009
<i>Pikh</i>	NBS-LRR	NA	Rai <i>et al.</i> , 2009
<i>p21*</i>	Proline-containing protein	NA	Fukuoka <i>et al.</i> , 2009
<i>Pb1*</i>	NBS-LRR	NA	Hayashi <i>et al.</i> , 2009

*Cloned blast resistance QTLs.

NA, not available; NBS-LRR, nucleotide-binding site and leucine-rich repeat.

Fig. 2 Phylogenetic relationship of the cloned rice nucleotide-binding site and leucine-rich repeat (NBS-LRR) proteins conferring resistance to *Magnaporthe oryzae*. Protein sequences were collected from the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/>) and used for alignment with CLUSTALW 2.0 (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). The neighbour-joining tree was constructed by the program MEGA 4.0 with the bootstrap test (bootstrap replications, 1000; random seeds, 24 054), and a bootstrap value of less than 40% for each branch was set as a cut-off.

Two candidate NBS-LRR genes (*Pikm1-TS* and *Pikm2-TS*) were identified at the *Pikm* locus. Complementary tests showed that transgenic plants carrying either of the NBS-LRR genes did not confer *Pikm* resistance specificity. *Pikm* resistance was complemented only when the two candidate genes were present in the same plants, suggesting that *Pikm*-mediated resistance is controlled by two adjacent NBS-LRR genes. A similar case was also found at the *Pi5* locus (Lee *et al.*, 2009). Transformation experiments have shown that both NBS-LRR genes, *Pi5-1* and *Pi5-2*, are required for *Pi5*-mediated resistance. Similarly, a new study has shown that, in *Arabidopsis*, both adjacent NBS-LRR genes,

RRS1 and *RPS4*, are required for resistance to *R. solanacearum* and *P. syringae* pv. *tomato* strain DC3000 expressing *avrRps4* (Narusaka *et al.*, 2009). Understanding how these two R proteins work together, and which one interacts with the cognate Avr protein, will certainly provide new insights into the molecular basis of this type of R-gene-mediated resistance.

It is noteworthy that *Pi-d2* is the only non-NBS-LRR-encoding R gene in rice cloned to date. The dominant R gene was identified in the Chinese rice variety Digu, which confers resistance to all 156 blast isolates collected from China and Japan, and has been widely used in breeding programmes in China as a blast-resistant donor (Chen *et al.*, 2006). *Pi-d2* encodes a receptor-like kinase protein with a predicted extracellular domain of a bulb-type, mannose-specific binding lectin (B-lectin) and an intracellular serine–threonine kinase domain. The lectin domains in *Pi-d2* contain predicted hydrophobic regions that form a structural pocket predicted for ligand binding. These results suggest that *Pi-d2* may detect its cognate Avr protein via a direct mechanism. Isolation of the Avr gene of the *Pi-d2* gene in *M. oryzae* in the future will shed light onto the *Pi-d2*-mediated broad-spectrum resistance to rice blast.

CLONING AND CHARACTERIZATION OF BLAST QTLs

Because partial resistant cultivars confer moderate and non-race-specific resistance to rice blast, their resistance is generally more durable and broad spectrum in natural conditions (Skamnioti and Gurr, 2009). For example, a local moderate-resistant cultivar called Acuce in Yunnan Province, China, has been cultivated for over 100 years and is still being grown by farmers in blast conducive regions (Y. Zhu, Yunnan Agricultural University, Kunming, China). Therefore, the identification and cloning of

Table 2 The characteristics of the cloned avirulence (Avr) effector genes from *Magnaporthe oryzae*.

Avr gene	Encoding protein	Cognate R gene	Reference
<i>PWL1</i>	Glycine-rich, hydrophilic protein, secreted protein	NA	Kang <i>et al.</i> , 1995; Sweigard <i>et al.</i> , 1995
<i>PWL2</i>	Glycine-rich, hydrophilic protein, secreted protein	NA	Kang <i>et al.</i> , 1995; Sweigard <i>et al.</i> , 1995
<i>AvrPi-ta</i>	Secreted protein	<i>Pi-ta</i>	Orbach <i>et al.</i> , 2000
<i>Avr1-CO39</i>	Secreted protein	<i>Pi-CO39</i>	Leong, 2008
<i>ACE1</i>	Polyketide synthase /peptide synthetase	<i>Pi33</i>	Böhnert <i>et al.</i> , 2004
<i>AvrPiz-t</i>	Secreted protein	<i>Piz-t</i>	Li <i>et al.</i> , 2009
<i>AvrPia</i>	Secreted protein	<i>Pia</i>	Miki <i>et al.</i> , 2009; Yoshida <i>et al.</i> , 2009
<i>AvrPii</i>	Secreted protein	<i>Pii</i>	Yoshida <i>et al.</i> , 2009
<i>AvrPik/km/kp</i>	Secreted protein	<i>Pik/km/kp</i>	Yoshida <i>et al.</i> , 2009

NA, not available.

resistance QTLs will provide new strategies to effectively control rice blast. The first set of blast QTLs was identified in the durably resistant upland cultivar Moroberekan using the interval mapping method (Wang *et al.*, 1994). Nine major QTLs have been defined with molecular markers, i.e. *Pb1* (Fujii *et al.*, 2000), *pi21* (Fukuoka and Okuno, 2001; Fukuoka *et al.*, 2007), *Pi34* (Zenbayashi *et al.*, 2002; Zenbayashi-Sawata *et al.*, 2007), *Pif* (Monosi *et al.*, 2004), *Pikur1* and *Pikur2* (Monosi *et al.*, 2004), *Pi-se1* (Wisser *et al.*, 2005), *Pi35* (Nguyen *et al.*, 2006) and *Pikahei-1(t)* (Miyamoto *et al.*, 2001; Xu *et al.*, 2008) (Fig. 1). Fukuoka and Okuno (2001) identified the recessive partial (non-race-specific) resistance gene *pi21* located on chromosome 4 from an upland cultivar Owarihatamochi. Recently, this group reported the first cloned partial R gene or QTL (Fukuoka *et al.*, 2007, 2009). *Pi21* encodes a proline-rich protein that includes a putative heavy metal-binding domain and putative protein–protein interaction motifs. Interestingly, wild-type *Pi21* appears to slow plant defence responses, and resistant *pi21*-containing plants carrying a deletion in its proline-rich motif inhibit this slowing. Another major QTL, *Pb1*, has been cloned recently from an *indica* rice cultivar Mudan that has been cultivated for over 20 years (Fujii *et al.*, 2000; Hayashi *et al.*, 2009). It confers more effective resistance at adult stages, especially for panicle blast. The *Pb1* gene also encodes a coiled-coil NBS-LRR protein. It will be interesting to determine whether these QTLs can be recognized by a single or multiple Avr gene, and what the mechanisms are for the activation of defence pathways following blast infection.

IDENTIFICATION OF GENES DOWNSTREAM OF R GENES

The identification of genes downstream of R genes is essential for the dissection of the resistance pathway. This has been a challenging task because of the lack of suitable protein–protein interaction assay methods for NSB-LRR R proteins and gene function redundancy and lethality when mutagenesis is used. Using *Pi-ta* as the bait in yeast two-hybrid (Y2H) screens, an

Avr–*Pi-ta* interacting protein, named AVI3, was identified from a blast-infected cDNA library of the *Pi-ta*-carrying cultivar Kay (Y. Jia, USDA-ARS, Stuttgart, AR, USA). This gene encodes a putative transcription factor and is induced by a fungal elicitor in rice suspension cells. The role of AVI3 in *Pi-ta*-mediated signal pathways is being investigated. Using a mutagenesis approach, a *Pi-ta*-expressing susceptible mutant was identified, named *Ptr(t)*, that is more probably specific to *Pi-ta*-mediated signal recognition because it is not required for other R genes (Jia and Martin, 2008). Genetic analysis has indicated that *Ptr(t)* segregates as a single dominant nuclear gene and is linked with *Pi-ta*. Genetic analysis has shown that the *Pi-ta* and *Ptr(t)* genes are located within a nine-megabase region. The cloning of *Ptr(t)* will be a significant advancement in the understanding of *Pi-ta*-mediated signal recognition and transduction.

CLONING OF AVR GENES

Pathogen effectors have been defined recently as all pathogen proteins and small molecules that alter host cell structure and function (Hogenhout *et al.*, 2009; de Wit and Stergiopoulos, 2009). Avr proteins are a special class of effectors that are recognized by corresponding R proteins, which lead to direct or indirect activation of host defence responses against pathogen invasion. During recent years, significant progress has been obtained in the identification of Avr effectors of the blast fungus, which has provided great insight into the understanding of the molecular mechanisms of fungal pathogenesis, and has also shed light onto the mechanisms of co-evolution between fungal effectors and host R proteins.

Among more than 40 identified *M. oryzae* Avr genes (Ma *et al.*, 2006), nine Avr genes have been cloned and characterized (Table 2). *PWL1* and *PWL2* were the first two cloned, which confer avirulence of the *Eleusine* and *Oryza* isolates, respectively, towards weeping lovegrass, *Eragrostis curvula* (Kang *et al.*, 1995; Sweigard *et al.*, 1995). Orbach *et al.* (2000) cloned the second rice blast Avr gene *AvrPi-ta*, which encodes a putative 223 amino acids containing secreted protein with a conserved

metalloprotease domain. Recently, using a map-based cloning strategy, Li *et al.* (2009) successfully cloned the *AvrPiz-t* gene, which encodes a putative secreted protein without any homologues in *M. oryzae* or other sequenced fungi. A recent whole-genome sequencing study by Yoshida *et al.* (2009) showed that *M. oryzae* isolate Ina168 contains an extra 1.68 Mb of DNA sequence that is absent from the sequenced strain 70-15. Most interestingly, a total of 318 candidate effector genes are predicted in this extra DNA. Sequence comparative and association genetic analysis identified three novel *Avr* genes, i.e. *AvrPia*, *AvrPii* and *AvrPik/km/kp*. All three were confirmed to be secreted proteins with a predicted signal peptide at their N-terminals. *AvrPia* and *AvrPik/km/kp* encode 85 amino acids and 113 amino acids with unknown functions, respectively. Another group also cloned *AvrPia* via a map-based cloning strategy from a gain of virulent mutant *Ina168m95-1*, which is virulent to rice cultivar Aichi-asahi harbouring the R gene *Pia* (Miki *et al.*, 2009). *AvrPii* is a 70-amino-acid peptide with a predicted motif-1 ([LI]x-AR[SE][DSE]) containing a conserved LxAR motif, which also appears in *AvrPiz-t*, and motif-2 ([RK]CxxCxxxxxxxxxxH), which is a zinc finger-like motif involved in protein–protein interaction. Interestingly, the predicted LxAR motif containing proteins represents a new large family of effectors in the rice blast genome (Yoshida *et al.*, 2009, B. Zhou, Zhejiang University, Hangzhou, China). Further elucidation of the function of this group of effectors will provide new information on how *M. oryzae* effectors are translocated to rice cells.

Using a map-based cloning approach, *Avr1-CO39* was delimited to a 1.05-kb region with several potential open reading frames (ORFs) (Farman and Leong, 1998; Farman *et al.*, 2002). Recently, this group reported that the 1.05-kb region contains eight predicted ORFs and that *Orf3* should be the functional *Avr1CO39* gene, which encodes 89 amino acids with a predicted signal sequence. They also found that *Avr1CO39* is transcribed only in plant cells, and that fusion with green fluorescent protein (GFP) shows reduced presence in resistance plant lines as a result of the activation of defence responses (Leong, 2008).

ACE1 is a unique *Avr* gene because it encodes a hybrid polyketide synthase/nonribosomal peptide synthetase (PKS-NRPS) (Böhnert *et al.*, 2004). The enzyme is probably involved in the biosynthesis of a polyketide (Collemare *et al.*, 2008). *ACE1* biosynthetic activity is required for its avirulence; however, the avirulence signal recognized by the cognate R gene *Pi33* in rice cultivars is not the *ACE1* protein, but the secondary metabolite synthesized by *ACE1* (Böhnert *et al.*, 2004). Interestingly, *ACE1* is appressorium-specific and is expressed in mature appressoria differentiated on plant leaves or artificial cellophane membranes during the penetration process (Fudal *et al.*, 2007). These results suggest that *ACE1* is connected to the onset of appressorium-mediated penetration, but does not enter directly into rice cells to trigger defence responses.

POPULATION DIVERSITY AND GENETIC INSTABILITY OF *Avr* GENES IN THE FIELD

A highly diverse genetic structure is a main character of the blast populations worldwide, and can be largely attributed to genetic instability and the loss of avirulence to R genes (Skamnioti and Gurr, 2009; Tharreau *et al.*, 2009). The frequent generation of new virulent races or pathotypes leads to the short life of many newly released cultivars. Recent studies on the variations of *Avr* genes have provided new insight into the mechanism of blast genetic variations and instability. Multiple genetic mutation events, including deletion (Orbach *et al.*, 2000), point mutations (Orbach *et al.*, 2000) and transposon insertion (Kang *et al.*, 2001), have been found to be the main driving force to the creation of new virulent races that break major R genes. For example, Orbach *et al.* (2000) found that a fragment deletion from intron 3 to exon 4 in the mutant strain CP983, several nonsense mutations (e.g. TGG1487TAG in the mutant strain CP918, TTA1736TGA in the mutant strain CP1615) and a missense mutation (e.g. GAA1718GGA in the mutant strain CP1635) led to a gain of virulence of the *AvrPi-ta* gene. In addition, transposon insertion usually leads to a gain of virulence for *Avr* genes through a change in gene expression level or pattern. Fudal *et al.* (2005) found that an insertion of a 1.9-kb MINE retrotransposon in the last *ACE1* exon led to a loss of *ACE1* avirulence and activated its virulence in the virulent isolate 2/0/3. Similar events were also found in *AvrPi-ta* (Zhou *et al.*, 2007) and *AvrPiz-t* (Li *et al.*, 2009). Close monitoring of the profiles of known *Avr* genes in the field will provide a reliable reference for the deployment of R genes in rice production.

INNATE IMMUNITY AND R GENE SIGNALLING

Plant-pathogenic bacteria and fungi suppress plant innate immunity and promote pathogenesis by injecting effector proteins into plant cells (Hogenhout *et al.*, 2009). These effectors can be generally divided into two main groups. One group, pathogen-associated molecular patterns (PAMPs), is recognized by pattern recognition receptors (PRRs) predominantly localized in the extracellular matrix. This recognition was previously known as basal defence, but is now referred to as PAMP-triggered immunity (PTI) (Block *et al.*, 2008; Jones and Dangl, 2006). The second group contains avirulence proteins that interact directly or indirectly with the cognate R proteins in the host. This recognition is associated with the well-known gene-for-gene hypothesis, and is now called effector-triggered immunity (ETI). Recent findings have shown that plants can rapidly develop effective ETI to inhibit pathogen growth when PTI is overcome by newly evolved pathogen effectors (Xiang *et al.*, 2008). At present, PTI-mediated defence has been well investigated in plant–bacteria pathosystems (Nicaise *et al.*, 2009), but

not in plant fungal diseases. Recently, the bacterial flagellin factor flg22 has been found to induce the rice immune response, which was perceived by rice PRR OsFLS2, the rice orthologue of AtFLS2 (Takai *et al.*, 2008; Takakura *et al.*, 2008). This result suggests that rice may have a similar PTI-mediated defence system, but how effective the system is to the defence of rice pathogens remains unknown.

In the model plant *Arabidopsis*, SGT1 and RAR1 have been shown to play a role in the ETI-mediated defence signalling pathway (Azevedo *et al.*, 2002). In rice, orthologues OsRAR1 and OsSGT1 associate with the rice small GTPase protein OsRac1 and are involved in resistance to bacterial blight and blast (Kawasaki *et al.*, 1999; Ono *et al.*, 2001; Suharsono *et al.*, 2002; Thao *et al.*, 2007; Wong *et al.*, 2007). Recently, the NBS-LRR R protein Pit has been found to interact directly with OsRac1, which leads to the activation of the ETI-mediated immune response (Kawano *et al.*, 2009). These results demonstrate that rice has a similar SGT1/RAR1-mediated signalling pathway to *Arabidopsis*, and the PTI and ETI defence pathways may be interconnected through membrane-associated proteins such as OsRac1.

FUTURE PERSPECTIVES

Rice continues to be the nutritional, financial and social lifeline for the majority of humanity, with its consumption growing dramatically in many parts of the world, including sub-Saharan Africa. Although intensive breeding and refined cultural practices have improved the rice yield considerably in the last several decades, many constraints will continue to limit sustainable rice production in most rice-growing regions. Among them, rice blast disease is considered to be the largest threat for stable rice production. As a result of the high level of pathogen instability in the field, breeders have battled rice blast by introducing new resistance genes into elite cultivars, but continue to be frustrated by the short life of newly released cultivars. Therefore, a deep and thorough understanding of how R and Avr proteins interact in the initial recognition process, the function of Avr effectors and their target host proteins in disease development, the exact structure of the pathogen population in the field and a clear genetic background of each rice cultivar is pivotal for the development of effective control methods for this important disease. Although impressive progress has been made in the above areas in recent years, many unanswered questions and challenges (listed below) lie ahead for the rice blast community.

- **How do R proteins interact with their cognate Avr proteins?** Although a direct interaction between AvrPi-ta and Pi-ta was revealed by far-western analysis several years ago (Jia *et al.*, 2000), no new evidence has been provided to demonstrate the direct interaction of the two proteins *in vivo*.

Using Piz-t or AvrPiz-t as bait in both Y2H and bimolecular fluorescence complementation (BiFC) analyses, no direct interaction between these two proteins was detected in our laboratory. Therefore, the determination of the interaction model for all nine cloned Avr and R gene pairs is a daunting task (Table 2), and new techniques should be applied to investigate the relationship between Avr and R proteins. Cloning of *Pikm* (Ashikawa *et al.*, 2008) and *Pi5* (Lee *et al.*, 2009) is exciting, but also raises a few critical questions as follows: Why are two highly homologous NBS-LRR proteins required for Pikm- and Pi5-mediated resistance? Are both proteins involved in the interaction with the cognate Avr protein? Is it possible that one of the R proteins functions as a 'decoy' in the newly proposed Decoy model (Van der Hoorn and Kamoun, 2008)?

- **What are the downstream genes of R genes?** Apart from the results obtained for the *Pi-ta* gene, no progress has been made in the identification of the genes required for other R-gene-mediated resistance. We used both Y2H and mutagenesis to identify downstream genes for the Pi9-, Pi2- and Piz-t-mediated pathways, but failed. New proteomic approaches, such as affinity binding, can be applied to pull down interacting proteins, as described by Nakashima *et al.* (2008).
- **What is the translocation mechanism of the blast Avr effectors?** The identification of the LxAR motif in both AvrPiz-t and AvrPii has provided clues on the possible translocation mechanism in *M. oryzae* (Li *et al.*, 2009; Yoshida *et al.*, 2009). To determine whether it has a similar function to the RXLR motif in the Avr proteins of *Phytophthora* species requires more experiments (Kamoun, 2006).
- **What are the targets of Avr proteins in rice cells and their functions in the host response to pathogen infection?** AVI3 is the only rice protein that interacts with AvrPi-ta. Its function in Pi-ta-mediated resistance is unknown. Using AvrPiz-t as the bait in Y2H screens, we identified eight interacting proteins from a rice cDNA library. Interestingly, four are involved in the ubiquitination pathway, suggesting a possible role of the ubiquitination-mediated pathway in the plant and fungal interaction (Birch *et al.*, 2009).
- **How to predict the durability of R genes based on pathogen population?** The breeding of new cultivars for a specific environment is a time-consuming and expensive process. The rapid breakdown of disease resistance of newly released cultivars has prompted breeders to seek ways in which to keep R genes effective for the long term. As more and more Avr and R gene pairs are cloned, it is possible to monitor the frequency of most Avr genes present in a field population and use the information to strategically deploy R genes in a specific environment. Large-scale and controlled field experiments are needed to test the feasibility of this approach.

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