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Recent advances in rice blast effector research

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To cause rice blast disease, the fungus *Magnaporthe oryzae* produces biotrophic invasive hyphae that secrete effectors at the host–pathogen interface. Effectors facilitate disease development, but some (avirulence effectors) also trigger the host's resistance gene-mediated hypersensitive response and block disease. The number of cloned *M. oryzae* avirulence effector genes has recently doubled, largely based on resequencing with a Japanese field isolate and association of avirulence activity with presence/absence polymorphisms in novel genes for secreted proteins. Effectors secreted by hyphae in rice cells accumulate in biotrophic interfacial complexes, and this property correlates with their translocation across plasma membrane into the rice cytoplasm. Interestingly, the translocated effectors moved into surrounding uninvaded cells, suggesting that effectors prepare host cells before the fungus enters them.

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Introduction

The filamentous ascomycetous fungus *Magnaporthe oryzae* (previously *M. grisea*) includes the *Oryza* population responsible for the most important disease of rice (*Oryza sativa*), as well as distinct populations adapted for infecting other agricultural crops such as wheat (*Triticum aestivum*) [1,2]. Through many centuries of rice cultivation, the global *Oryza* population has evolved in response to the regional histories of rice cultivar deployment in diverse rice-growing regions [3]. The result is a complex population structure with hundreds of races, which differ in content of avirulence (AVR) effectors that trigger hypersensitive resistance (HR) in varieties with corresponding resistance (*R*) genes. In rice, ~85 blast *R* genes have been identified so far [4••], but individual *R* genes lose effectiveness within one to two years after they are deployed in the field, because of the high levels of AVR effector variability in the fungus. Wheat blast caused by *Triticum* isolates is a recently emerged disease, first reported in Brazil in 1985 [5]. Although wheat blast is currently restricted to South America, it now looms as a major threat to global wheat production. With the identification of *AVR*-like genes preventing wheat blast and of the first wheat blast *R* genes [6••], it is reasonable to predict that wheat blast will show AVR effector evolution as in rice blast.

This review is focused on blast AVR effector biology and on putative effectors in the complex *M. oryzae* secretome. Recent reviews cover the 13 blast *R* genes and 2 QTLs that have been cloned [7•], and current understanding of the mechanisms of *M. oryzae* pathogenesis [8,9], including recent insight on the critical role for autophagy during pathogenicity-related development [10].

A novel AVR gene expressed during appressorial penetration

Two properties of the *ACE1* gene, which confers AVR toward rice containing the *R* gene *Pi33*, make it unique among known eukaryotic *AVR* genes. First, *ACE1* encodes a 4035-amino acid cytoplasmic enzyme, a hybrid polyketide synthase–nonribosomal peptide synthetase (PKS–NRPS). ACE1 probably functions in the biosynthesis of a small molecule (secondary metabolite) that is the actual effector recognized by the Pi33 gene product [11]. Second, *ACE1* expression is tightly coupled to the onset of appressorium-mediated penetration of the host cuticle (a narrow peak of expression ~17 hours post-inoculation, hpi) [12]. Recently, it has been reported that *ACE1* is located in a secondary metabolism-related cluster of 15 coordinately regulated genes [13••]. This gene cluster contains a second PKS–NRPS gene, *SYN2*, and other genes potentially encoding enzymes that modify the secondary metabolite precursors produced by the ACE1 and SYN2 enzymes. Functional analysis of these genes has proven difficult. After repeated attempts, knockout mutants were only obtained for *SYN2* and one other gene, and these mutants were still fully virulent on rice lacking *Pi33* and still AVR on rice with *Pi33*. However, the AVR activity of *ACE1* and the exquisitely timed expression of the *ACE1* gene cluster suggest a role during appressorial penetration. The *M. oryzae* genome is highly enriched in secondary metabolism genes (with the highest number of PKS–NRPS genes in any fungus) [14], which suggests that other secondary metabolites may function as AVR effectors.

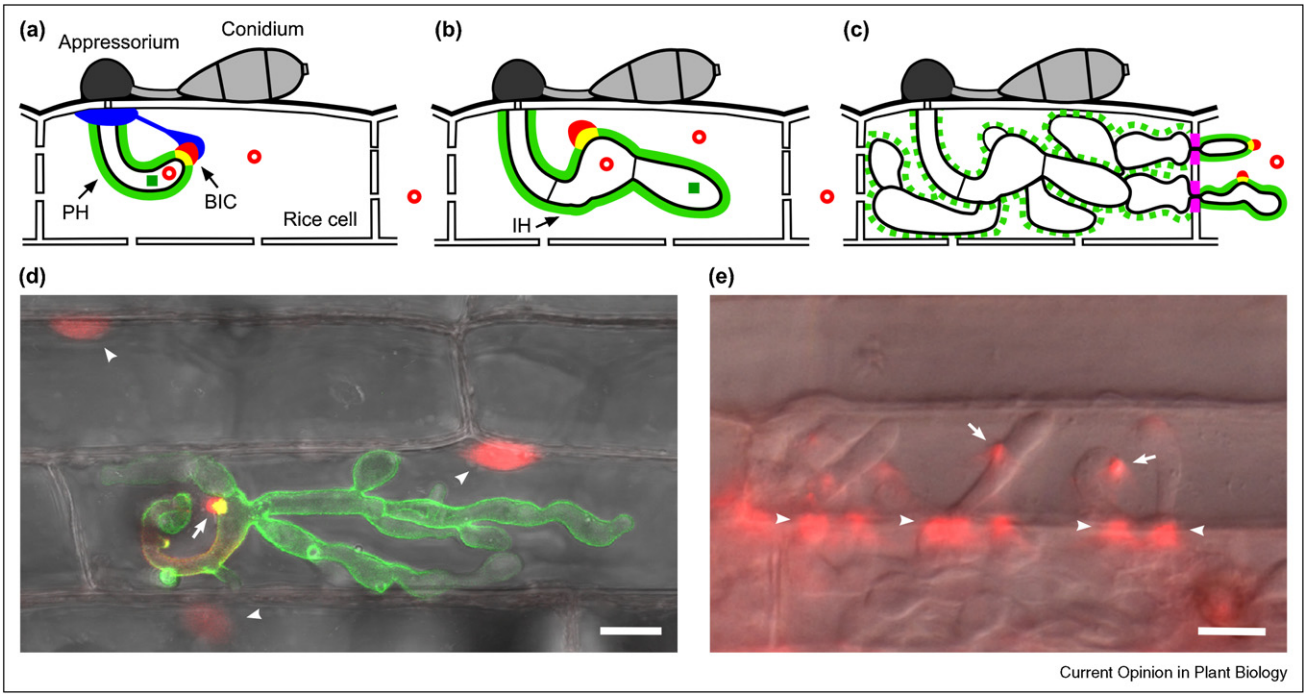
Secretion of AVR effector proteins by intracellular invasive hyphae

Ease of *M. oryzae* genetic transformation and of *in planta* live-cell imaging of blast biotrophic invasive hyphae (IH)

growing in rice sheath cells [15,16] has enabled direct observation of transgenic IH secreting AVR effector proteins linked to green (GFP) or red (mRFP) fluorescent proteins [17••,18••]. When fluorescently labeled effectors AVR-Pita1 [19•,20], PWL1 [21], and PWL2 [22] were expressed using their native promoters, they preferentially accumulated in the biotrophic interfacial complex (BIC), a novel interfacial structure associated with the first hyphal cells to invade the host cell (see Figure 1a–e). Using the effector promoter, the signal peptide-encoding sequence was interchangeable with the entire effector coding sequence in mediating GFP localization in BICs [17••]. It is currently unknown if preferential BIC accumulation involves differential effector gene expression, a specialized secretion mechanism, or both. It is also possible that 5′ mRNA sequences play a role in BIC targeting.

Mutational analysis of effector secretion mechanisms is a relatively new area in blast research. Knockout mutants lacking the *LHS1* gene, which encodes an ER chaperone functioning in protein secretion, were severely impaired in biotrophic invasion, but they showed only minor growth defects on artificial medium [23••], underscoring

Figure 1



Development of the biotrophic interfacial complex (BIC) and effector trafficking during biotrophic invasion of rice cells. **(a–c)** On the rice surface, a conidium germinates and produces a pressurized appressorium to mechanically breach the host cuticle and epidermal cell wall. Primary hyphae (PH) and then bulbous invasive hyphae (IH) invade living rice cells while separated from the host cytoplasm (symplast) and extracellular space (bulk apoplast) by plant-derived extrainvasive-hyphal membrane (EIHM) [15]. In fully susceptible interactions, IH successively invade living host cells, but invaded cells eventually die as they fill with fungus. Schematic diagrams, not drawn to scale, depict sheath cell invasion by transgenic fungal strains expressing effector proteins (or BAS1) fused to red fluorescent protein (mRFP) and BAS4 protein fused to green fluorescent protein (GFP). (a) Initially (22–25 hpi), a filamentous PH secretes chimeric effector:mRFP (red rings), which preferentially accumulates in the BIC at the hyphal tip (red dome). BAS4:GFP (green squares) is secreted and retained in the EIHM compartment surrounding the PH (green solid line) [18••]. Rice cytoplasm (blue) showed dynamic accumulations near the BIC, and these accumulations were often tethered to cytoplasm beneath the penetration site [17••]. (b) At 26–30 hpi, the PH differentiates into bulbous IH, which exhibit pseudohyphal growth, and the BIC (red dome) is left behind beside the first IH cell. Fluorescence recovery after photobleaching experiments showed that fluorescent PWL2 continued to be secreted into side BICs as IH grew elsewhere in the cell. Two BIC-localized secreted proteins, PWL2 and BAS1 (red rings in panels a–c) were shown to be translocated to the cytoplasm of the invaded cell [17••], and both moved into adjacent uninvaded neighbors. In contrast, GFP-labeled BAS4 protein outlined the subsequently formed IH, but was not translocated to the host cytoplasm. It has been hypothesized, but not proven, that BICs mediate effector translocation into host cells [17••]. (c) To move into neighbor cells at 36–40 hpi, IH swell slightly, form highly constricted pegs to cross the cell wall, initially grow as filamentous IH and then enlarge again into IH. The two stages of BIC development (red domes) are repeated for each hypha that enters a living host cell. **(d)** This confocal projection image shows an IH (at 30 hpi) expressing BAS4:GFP, and effector PWL2 fused to mCherry (red, a brighter variant of mRFP) that had been engineered with a C-terminal viral nuclear localization signal (NLS). Fluorescently labeled PWL2 (red) preferentially accumulated in the BIC (arrow). Observation of translocated PWL2 in invaded cells and in uninvaded neighbors was facilitated by experimentally targeting it to host nuclei (arrowheads) with the added NLS. Scale bar = 10 μm. **(e)** IH transformed to express BAS2:mRFP (red) were imaged after they had moved into a second-invaded cell at 36 hpi. BAS2:mRFP fusion protein accumulated in BICs (arrows) and at cell wall crossing points (arrowheads, purple in panel c). (Image courtesy of MC Giraldo.) Scale bar = 10 μm.

the importance of secreted proteins for disease development. The *lhs1*$^{-}$ mutants also failed to accumulate effectors in BICs, and to develop *Pita*-mediated HR, confirming that AVR effectors enter the classical ER pathway for secretion. A previous study of mutants lacking the *APT2* gene, encoding a Golgi-localized P-type ATPase, indicated that effectors might be secreted through an exocytotic pathway that is specific to plant infection [24]. Together, these studies suggest that the effector secretion pathway diverges after entry into the ER. Many interesting questions remain on *in planta* effector secretion in rice blast disease.

Characterization of new AVR effectors

Four blast AVR effectors have been described in the last year (Table 1). *AvrPiz-t* was identified by map-based cloning [25••], and the others were identified by association mapping. After failing to find AVR candidates among 1032 putative secreted proteins from sequenced *M. oryzae* strain 70-15 [26], Yoshida *et al.* [27••] resequenced Ina168, a Japanese rice isolate with nine *AVR* genes. Amazingly, 1.68 Mb of Ina168 genome sequence failed to align with the 70–15 genome, including sequences encoding 316 small putative secreted (PEX) proteins. Associations between particular *PEX* genes and AVR phenotypes identified *AVR-Pia*, *AVR-Pii*, and *AVR-Pik/km/kp* [27••]. Independently, Miki *et al.* [28••] cloned *AVR-Pia* by the identification of sequences that had been deleted in a spontaneous *avr-pia*$^{-}$ mutant lacking AVR activity.

The four new effectors (Table 1) continue the theme that most effectors are small novel secreted proteins generally lacking homology to known proteins [29–31]. It has been difficult to establish virulence functions for blast effectors because knockout mutants fail to show phenotypes. However, AvrPiz-t suppresses BAX-mediated programed cell death in tobacco leaves, suggesting that it functions in blocking plant immunity [25••]. As with *AVR-Pita1* [32], expression of *AVR-Pia*, *AVR-Pii*, and *AVR-Pik/km/kp* in rice with the cognate *R* gene suggests they function after translocation into the rice cytoplasm [27••]. *AVR-Pik/km/kp* is interesting because it confers AVR activity toward three *R* genes (*Pik*, *Pikm*, and *Pikp*), and *Pikm* is one of two blast *R* genes requiring two linked NBS-LRR genes for activity [7•,33•]. The AVR-Pii protein stands out in having two motifs that are both conserved in three other predicted *M. oryzae* proteins [27••]. The LxAR motif

Table 1

Properties of proteinaceous blast AVR effectors and BAS proteins

Gene name[a]	*R* gene	aa (cys)[b]	Alleles or related genes/proteins (AVR activity +/–)[c]	Motifs/function/localization[d]	Reference
PWL2 (MGG_04301.6; MGG_13863.6)	Unknown	145 (2)	Gene family: *PWL1* (+), *PWL3* (–), *PWL4* (–); MGG_07398.6	–/–/BIC, translocated to host cytoplasm	[17••,21,22]
AVR-Pita1 (MGG_15370.6)	*Pita* (cloned)	224 (9)	Gene family: *AVR-Pita2* (+), *AVR-Pita3* (–); MGG_14981.6 and 6 other genes	Zinc-metalloprotease motif/–/BIC, functions in host cytoplasm	[17••,19•,32]
AVR1-CO39	*PiCO39(t)*	89 (3)	None	–/–/–	[39]
AvrPiz-t	*Piz-t* (cloned)	108 (5)	None	LxAR/suppresses Bax-PCD/–	[25••,47]
AVR-Pia	*Pia*	85 (2)	None	–/–/functions in host cytoplasm	[27••,28••]
AVR-Pii	*Pii*	70 (3)	XP_366338.2, XP_001407225.1, XP_364190.1	(LI)xAR[SE][DSE] and [RK]CxxCx$_{12}$H/–/ functions in host cytoplasm	[27••]
AVR-Pik/km/kp	*Pikm* (cloned)	113 (3)	Five alleles (A–E)	–/–/functions in host cytoplasm	[27••,33•,48]
BAS1 (MGG_04795.6)	Unknown	115 (0)	None	–/–/BIC, translocated to host cytoplasm	[17••,18••]
BAS2 (MGG_09693.6)	Unknown	102 (6)	MGG_07969.6, MGG_07749.6,	–/–/BIC, cell wall crossing points	[18••]
BAS3 (MGG_11610.6)	Unknown	113 (10)	None	–/–/BIC, cell wall crossing points	[18••]
BAS4 (MGG_10914.6)	Unknown	102 (8)	MGG_02154.6	–/–/EIHM matrix	[17••,18••]

[a] MGG_numbers are from the genome sequence release 6 (http://www.broad.mit.edu/annotation/genome/magnaporthe_grisea/MultiHome.html).
[b] Number of amino acids (aa) and cysteine (cys) residues in the predicted protein are listed. Most of the known blast AVR effectors are low in cysteine residues and thus differ from many known apoplastic effectors that contain two or more pairs of cysteines that could form disulfide bonds.
[c] Only *M. oryzae* genes/proteins are listed. MGG_ or XP_numbers were identified by BLAST searches of the *Magnaporthe grisea* protein database (http://www.broadinstitute.org/annotation/genome/magnaporthe_grisea/Blast.html?sp=Sblastp; *E*-value < 1e−3) or NCBI protein database (*E*-value < 0.02; [27••]), respectively. BLAST searches of NCBI protein database identified proteins related to the predicted mature AVR-Pita1 protease in *Verticillium albo-atrum* (*E*-value = 5e−12) and many other fungi, and to BAS2 in *Pyrenophora tritici-repentis* (*E*-value = 6e−21) and nine other fungi. Presence (+) or absence (–) of AVR activity is indicated.
[d] 'Functions in host cytoplasm' indicates that transient (and sometimes conditional stable) expression in the host cytoplasm induced HR in rice with the corresponding *R* gene [27••,32]. 'Translocated to host cytoplasm' indicates direct microscopic observation in the host cytoplasm [17••]. Minus (–) signs indicate the lack of experimental evidence.

([LI]xAR[SE][DSE]) is also loosely conserved in *AvrPiz-t* and other effectors, and has been suggested as a translocation motif based on sequence comparisons ([7•], B Zhou, unpublished data). The second motif ([RK]CxxCxxxxxxxxxxxH) in AVR-Pii, shared by 15 candidate effectors, resembles a C2H2 zinc finger motif involved in protein–protein interactions. Perhaps AVR-Pii and related proteins interact directly with host targets.

Genetic variation of blast *AVR* genes

All cloned blast *AVR* genes are closely associated with diverse repeated sequences, including active transposable elements (TEs), and among the >40 mapped and cloned *AVR* genes, several appear to be located near telomeres [20,27••,34]. Subtelomeric *AVR-Pita1* undergoes frequent mutational events leading to loss of avirulence. During subculture on artificial medium, *AVR-Pita1* undergoes spontaneous deletions, nucleotide substitutions, and inactivation by TE insertions, and similar events are recovered from the field [20,35,36]. Studying a set of 21 field isolates, *AVR-Pita1* showed by far the highest levels of nucleotide diversity among 1032 secreted proteins [27••]. Field isolates also show copy number polymorphisms of *AVR-Pita1*, with individual strains containing zero to three copies. *AVR-Pita1*, cloned from an *Oryza* isolate, defines a gene family together with *AVR-Pita2* and *AVR-Pita3*, which are distributed across the host species-specialized populations [19•]. The *avr-pita1*$^-$ and *AVR-Pita3* alleles lacking AVR activity contain protease motifs, suggesting loss of recognition without loss of virulence function [19•,36]. Only rare rice pathogens lack all three family members. These results are consistent with the *AVR-Pita* genes playing a still unknown role during biotrophic invasion.

Among the newly cloned genes, *AVR-Pik/km/kp* showed presence/absence polymorphisms as well as an excess of nonsynonymous nucleotide substitutions characteristic of positive selection [27••]. This *AVR* gene occurs as five alleles collectively differing by five nonsynonymous polymorphic sites, and the D allele has AVR activity. So far, *AVR-Pia* and *AVR-Pii* (appears subtelomeric in two different studies) show only gain/loss mutations [27••]. Alleles of *AvrPiz-t* that lacked AVR function had either insertion of a TE, as observed for some non-AVR-conferring *AVR-Pita1* and *ACE1* alleles [35–37], or a single nonsynonymous nucleotide substitution [25••]. Clearly, repetitive sequences in *M. oryzae* contribute to rapid evolution of host specificity by diverse mutational events, which poses a challenge in achieving durable blast resistance.

Effectors and host species specificity within *M. oryzae*

Effectors are also involved in determining *M. oryzae* host species specificity. The first cloned *AVR* effector, *PWL2* (Table 1), prevents *M. oryzae* strains from infecting weeping lovegrass (*Eragrostis curvula*) [21,22]. *PWL2* from *Oryza* isolates forms a gene family together with *AVR* allele *PWL1* (78% nucleotide identity with *PWL2*) and the non-*AVR* alleles *PWL3* (63% nucleotide identity) from *Eleusine* isolates (infecting finger millet, *Eleusine coracana*), and *PWL4* (65% nucleotide identity) from *Eragrostis* isolates [21]. *AVR1-CO39*, which functions as an *AVR* gene in rice (Table 1), is broadly present in *M. oryzae* populations adapted to other host species. This gene appears to have been gained by the ancestral *M. oryzae* isolate, and then lost from the *Oryza* population, apparently due to a TE-mediated deletion event [38–40].

Tosa *et al.* [41] have identified several *AVR* effector-like genes that control host species specificity. For example, five genes independently block infection of wheat: *PWT1* and *PWT2* from closely related *Oryza* and *Setaria* isolates (infecting foxtail millet, *Setaria italica*); *PWT3* and *PWT4* from *Avena* isolates (infecting oat, *Avena sativa*); and *PWT5* from *Oryza* isolates [41]. Differentiation of host species-adapted strains by *AVR* effector genes is consistent with cytological investigations showing that nonadapted strains often induce post-penetration HR-like responses that are characteristic of effector-induced HR in incompatible interactions of adapted strains [42•,43,44•]. The results suggest that host shifts, and emergence of new diseases like wheat blast, could result from loss of a small number of *AVR* genes.

An abundance of putative secreted blast fungal proteins

The predicted *M. oryzae* secretome has ranged from 7% (739 putative secreted proteins) of all predicted proteins in the original analysis [26] to 12% (1546 putative secreted proteins) after reanalysis using a different prediction regime [45••]. Recognizing that different prediction algorithms result in different candidates, Choi *et al.* [46••] developed a secreted protein prediction pipeline that incorporates results from nine different programs. They reported 2470 *M. oryzae* candidate secreted proteins, 22% of the predicted proteome. As an additional complication, the minimum length threshold for predicted proteins is critical for identifying effector candidates. To identify Ina168 effector candidates, Yoshida *et al.* [27••] used a minimum length threshold of >50 amino acids (aa) (compared to a threshold of >100 aa in other studies). Indeed, *AVR-Pia* (85 aa) and *AVR-Pii* (70 aa) would not have been identified with the higher threshold (Table 1). Clearly, the *M. oryzae* secretome is complex and provides enormous challenges for sorting through putative secreted proteins to identify real effectors.

A common characteristic of effectors across diverse pathosystems is that they have evolved at accelerated rates compared to most genes in the genome, presumably due to a coevolutionary arms race between the pathogen and its host [29–31]. Most *M. oryzae* secreted protein genes fail

to show signs of positive selection [27••]. EcoTilling experiments to detect nucleotide polymorphisms for 1032 predicted secreted protein genes using 46 diverse *Oryza* isolates showed that only 22% contained nucleotide substitutions. PCR-detected presence/absence polymorphisms were more common (38% were polymorphic among 21 isolates). Although the targeted *AVR* effectors were not present in this set of genes, *AVR-Pita1*, included as a control, showed the expected high levels of nucleotide substitutions and presence/absence polymorphism [27••]. This study showed that most predicted secreted proteins are conserved in *Oryza* strains, and that extensive polymorphisms remain a good predictor of AVR effectors.

By another criterion, biotrophic invasion-specific expression, novel secreted proteins predicted in the 70-15 genome sequence are strong effector candidates. Using a simple procedure to purify rice sheath tissue with IH growing in first-invaded epidermal cells, Mosquera *et al.* [18••] identified many secreted protein genes that are expressed during biotrophic invasion but not during culture *in vitro*. Four of these, named biotrophy-associated secreted (BAS) proteins one to four, were expressed in fungal transformants as fluorescent fusion proteins and confirmed to be specifically expressed and secreted during biotrophic invasion of rice cells. Knockout mutants for each failed to identify a phenotype, but detailed *in vivo* secretion analyses showed distinct localization patterns that provided clues to their functions ([18••]; MC Giraldo *et al.*, unpublished data). BAS1 showed preferential BIC accumulation characteristic of known AVR effectors, as well as translocation into the rice cytoplasm (see below). This protein might function similarly to AVR effectors. BAS2 (Figure 1e) and BAS3 accumulated in BICs and localized at the rice cell wall where IH had crossed into neighboring cells, perhaps providing clues as to how the fungus might seek out and use pit fields (clusters of plasmodesmata) for moving into neighbor cells [15]. BAS4 is a potential matrix protein accumulating between the extrainvasive-hyphal membrane (EIHM) and the IH cell wall. Assuming the BAS proteins are effectors, these studies support the suggestion that not all effectors target the host immune response [30]. Instead some may be involved in altering plant components required for biotrophic invasion.

Effector translocation and cell-to-cell movement

When fungal transformants secreted fluorescent PWL2 and BAS1 fusion proteins during epidermal cell invasion, these fluorescent proteins were observed in BICs and in the rice cytoplasm, demonstrating translocation across the host plasma membrane (Figure 1) [17••]. In the same experiments, the BAS4 putative matrix protein was observed surrounding the IH in the EIHM compartment, and was not translocated to the host cytoplasm. The translocated cytoplasmic fluorescence was faint, especially compared to fluorescence that had accumulated in the BIC. Observation of translocated fluorescent effectors was facilitated by shrinking the rice cytoplasm away from the cell wall through plasmolysis, or by experimentally targeting the effectors to host nuclei (Figure 1d). A sensitive *in vivo* translocation assay [17••] is now available to facilitate studies of translocation mechanisms and identification of effector properties that enable translocation. In particular, the translocation assay will enable mutational analyses of putative translocation motifs, such as LxAR [7•]. Identification of a blast effector translocation motif might allow genome database searches for additional effector candidates, as demonstrated for oomycete systems based on the conserved oomycete amino acid translocation motif (RXLR-dEER) [29,30].

Interestingly, fluorescent effector protein that reached the rice cytoplasm moved to neighbor cells before invasion by the fungus (Figure 1d), raising the possibility that effectors prepare rice cells before fungal invasion [17••]. This putative cell-to-cell trafficking of effectors depended on both rice cell type and on effector protein size, suggesting that effectors might be moving symplastically through plasmodesmata.

Future challenges

Half of the characterized blast *AVR* effector genes are not present in the assembled *M. oryzae* 70-15 genome, and gain/loss events are a major factor in AVR effector evolution. Therefore, multiple *Oryza* field isolates must be sequenced to fully capture the complex pathogen secretome and identify more blast AVR effectors. Accelerated gene evolution and biotrophic invasion-specific expression remain the best indicators for identifying AVR effectors. For practical purposes, it is important to clone the *AVR* effector genes that correspond to *R* genes available to rice breeders for the control of rice blast disease. Understanding *AVR* gene composition and dynamics in populations from diverse rice-growing regions will direct breeders to the best *R* gene combinations for local disease control.

From initial genetic analyses, it appears that *AVR* effector gene evolution could result in *M. oryzae* host shifts leading to emerging diseases such as wheat blast. Genome sequencing of wheat blast strains is needed. Since house-keeping genes appear to be conserved among the host species-adapted populations, genome comparisons should immediately identify putative wheat blast effectors. Understanding AVR effectors more broadly within the host species-adapted populations may identify novel *R* genes with utility for controlling rice and wheat blast diseases.

Much remains to be learned about *M. oryzae* effector functions during biotrophic invasion, including understanding host targets and *R* gene interactions. Apparent

functional redundancy of effectors provides challenges to identifying virulence functions. Double and triple knockout mutants may help in detecting virulence phenotypes. High-throughput secretion studies will divide BAS proteins into localization categories (BAS1 effector type, BAS2 wall crossing type, and BAS4 matrix protein type), which should be useful to guide further mutant analyses. Robust assays are now available for understanding effector secretion, translocation, and cell-to-cell trafficking [17••]. Identifying and mutating motifs necessary for BIC accumulation will determine if BICs have a role in effector translocation as hypothesized. Identification of nucleotide or amino acid sequence motifs that are critical for effector secretion, translocation, or cell-to-cell movement would perhaps provide a bioinformatic tool for *ab initio* identification of blast effectors.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Couch BC, Fudal I, Lebrun M-H, Tharreau D, Valent B, van Kim P, Notteghem J-L, Kohn LM: **Origins of host-specific populations of the blast pathogen *Magnaporthe oryzae* in crop domestication with subsequent expansion of pandemic clones on rice and weeds of rice**. *Genetics* 2005, **170**:613-630.

2. Couch BC, Kohn LM: **A multilocus gene genealogy concordant with host preference indicates segregation of a new species, *Magnaporthe oryzae*, from *M. grisea***. *Mycologia* 2002, **94**:683-693.

3. Wang G-L, Valent B (Eds): *Advances in Genetics, Genomics and Control of Rice Blast Disease*. New York: Springer Science and Business Media; 2009.

4. • Ballini E, Morel J-B, Droc G, Price A, Courtois B, Notteghem J-L, Tharreau D: **A genome-wide meta-analysis of rice blast resistance genes and quantitative trait loci provides new insights into partial and complete resistance**. *Mol Plant–Microbe Interact* 2008, **21**:859-868.
A comprehensive analysis of the rice blast resistance literature identified 85 major *R* genes and ~350 QTLs. These resistance loci were placed on the rice map and all data were made available in the OrygenesDB database. The authors used meta-analysis to accurately identify QTLs reported independently in separate studies. This paper includes an interesting discussion of properties of blast *R* genes relative to current hypotheses on *R* gene-based strategies for durable disease resistance.

5. Urashima AS, Igarashi S, Kato H: **Host range, mating type and fertility of *Pyricularia grisea* from wheat in Brazil**. *Plant Dis* 1993, **77**:1211-1216.

6. •• Zhan SW, Mayama S, Tosa Y: **Identification of two genes for resistance to *Triticum* isolates of *Magnaporthe oryzae* in wheat**. *Genome* 2008, **51**:216-221.
This first report of wheat *R* genes controlling wheat blast disease adds to the growing case that wheat blast is a gene-for-gene system similar to rice blast. This paper continues these authors' elegant genetic analyses of the fungus (see [41] and references therein), which identified multiple AVR effector genes that control the host species specificity in *M. oryzae*.

7. • Liu J, Wang X, Mitchell T, Hu Y, Liu X, Dai L, Wang G-L: **Recent progress and understanding of the molecular mechanisms of the rice and *Magnaporthe oryzae* interaction**. *Mol Plant Pathol* 2010, **11**:419-427.
This review focuses on the 13 major blast *R* genes that have been cloned, including genes corresponding to cloned AVR genes (Table 1). Two cloned quantitative trait loci (QTLs) are described, as well as cloned blast AVR genes.

8. Skamnioti P, Gurr SJ: **Against the grain: safeguarding rice from rice blast disease**. *Trends Biotechnol* 2009, **27**:141-150.

9. Wilson RA, Talbot NJ: **Under pressure: investigating the biology of plant infection by *Magnaporthe oryzae***. *Nat Rev Microbiol* 2009, **7**:185-195.

10. Talbot NJ, Kershaw MJ: **The emerging role of autophagy in plant pathogen attack and host defence**. *Curr Opin Plant Biol* 2009, **12**:444-450.

11. Böhnert HU, Fudal I, Dioh W, Tharreau D, Notteghem J-L, Lebrun M-H: **A putative polyketide synthase/peptide synthetase from *Magnaporthe grisea* signals pathogen attack to resistant rice**. *Plant Cell* 2004, **16**:2499-2513.

12. Fudal I, Collemare J, Böhnert HU, Melayah D, Lebrun M-H: **Expression of *Magnaporthe grisea* avirulence gene *ACE1* is connected to the initiation of appressorium-mediated penetration**. *Eukaryot Cell* 2007, **6**:546-554.

13. •• Collemare J, Pianfetti M, Houlle AE, Morin D, Camborde L, Gagey MJ, Barbisan C, Fudal I, Lebrun MH, Böhnert HU: ***Magnaporthe grisea* avirulence gene *ACE1* belongs to an infection-specific gene cluster involved in secondary metabolism**. *New Phytol* 2008, **179**:196-208.
It is not surprising that the *AVR* gene *ACE1* is located in a 15-member secondary metabolite-related gene cluster, since these genes are typically clustered. However, the additional coordinately regulated genes may function in the modification of the ACE1-precursor molecule to produce the active molecule, or they may function in the biosynthesis of an additional metabolite with a virulence function during penetration, or an AVR function toward some other *R* gene. Knockout mutants were obtained for two of the genes, but functional analysis of the others has proven difficult. This was true even when transforming an *M. oryzae* delta-ku80 mutant, which mainly produces knockout mutants due to a defect in the nonhomologous end-joining system responsible for ectopic DNA integration. The authors propose that cluster-specific chromatin modification is involved in its precise expression during penetration.

14. Collemare J, Billard A, Böhnert HU, Lebrun M-H: **Biosynthesis of secondary metabolites in the rice blast fungus *Magnaporthe grisea*: the role of hybrid PKS–NRPS in pathogenicity**. *Mycol Res* 2008, **112**:207-215.

15. Kankanala P, Czymmek K, Valent B: **Roles for rice membrane dynamics and plasmodesmata during biotrophic invasion by the blast fungus**. *Plant Cell* 2007, **19**:706-724.

16. Koga H, Dohi K, Nakayachi O, Mori M: **A novel inoculation method of *Magnaporthe grisea* for cytological observation of the infection process using intact leaf sheaths of rice plants**. *Physiol Mol Plant Pathol* 2004, **64**:67-72.

17. •• Khang CH, Berruyer R, Giraldo MC, Kankanala P, Park S-Y, Czymmek K, Kang S, Valent B: **Translocation of *Magnaporthe oryzae* effectors into rice cells and their subsequent cell-to-cell movement**. *Plant Cell* 2010, Published online, April 30, 2010; doi:10.1105/tpc.109.069666.
This paper continues in planta characterization of biotrophic IH [15,18••] by using live-cell imaging of fungal transformants secreting fluorescent effector fusion proteins as they invade rice sheath epidermal cells. Secreted fluorescent effectors preferentially accumulated in biotrophic interfacial complexes (BICs) at the IH–rice cell interface (Figure 1). Extensive quantitative analyses of interactions at hundreds of independent infection sites showed that BIC-localized PWL2 and BAS1 proteins were translocated to the cytoplasm of invaded rice cells. Based on this correlation, BICs were hypothesized to be involved in effector translocation, but this remains to be proven. A sensitive translocation assay, involving experimental targeting of the fluorescent effectors to the rice nucleus (Figure 1d), will allow future identification of translocation motifs for blast effectors. An additional exciting result from this study is that effector fusion proteins that reached the cytoplasm of invaded rice cells

moved into surrounding uninvaded rice cells, possibly preparing them before fungal invasion.

18. Mosquera G, Giraldo MC, Khang CH, Coughlan S, Valent B:
•• **Interaction transcriptome analysis identifies *Magnaporthe oryzae* BAS1-4 as biotrophy-associated secreted proteins in rice blast disease**. *Plant Cell* 2009, **21**:1273-1290.
It has previously been difficult to identify fungal genes that are expressed by IH during biotrophic invasion of rice cells because infected tissues contain too little fungal RNA at the early stages of invasion. These researchers adopted the hand-trimming procedure used for preparing invaded rice sheath pieces for live-cell imaging [15,17••], and obtained infected tissue RNAs that were ~20% RNA from IH in first-invaded cells. Microarray analysis with the Agilent whole genome *M. oryzae* array identified many genes for novel biotrophy-associated secreted (BAS) proteins that were previously only predicted by genome sequencing. Four of these, *BAS1* to *BAS4*, defined three distinct *in planta* localization patterns (Figure 1). Using the same IH RNA-enriched infected tissue samples with rice microarrays identified many candidate rice effector-triggered-susceptibility genes that remain to be investigated.

19. Khang CH, Park S-Y, Lee Y-H, Valent B, Kang S: **Genome**
• **organization and evolution of the *AVR-Pita* avirulence gene family in the *Magnaporthe grisea* species complex**. *Mol Plant–Microbe Interact* 2008, **21**:658-670.
This study describes the *AVR-Pita* gene family, which is unique to the *M. grisea* species complex, including *M. oryzae* and *M. grisea* (the fungal population infecting crabgrass, *Digitaria sanguinalis*, and related grasses). *AVR-Pita* (renamed *AVR-Pita1)* and *AVR-Pita2* (92% amino acid identity, confers AVR) occur in both *M. oryzae* and *M. grisea*, and *AVR-Pita3* (72% identity, non-AVR paralog) is restricted to *M. oryzae*. *AVR-Pita1* and *AVR-Pita2* were often located near telomeres, and all family members showed close association with diverse transposable elements.

20. Orbach MJ, Farrall L, Sweigard JA, Chumley FG, Valent B: **The fungal avirulence gene *AVR-Pita* determines efficacy for the rice blast resistance gene *Pi-ta***. *Plant Cell* 2000, **12**:2019-2032.

21. Kang S, Sweigard JA, Valent B: **The *PWL* host specificity gene family in the blast fungus *Magnaporthe grisea***. *Mol Plant–Microbe Interact* 1995, **8**:939-948.

22. Sweigard JA, Carroll AM, Kang S, Farrall L, Chumley FG, Valent B: **Identification, cloning, and characterization of *PWL2*, a gene for host species specificity in the rice blast fungus**. *Plant Cell* 1995, **7**:1221-1233.

23. Yi M, Chi M-H, Khang CH, Park S-Y, Kang S, Valent B, Lee Y-H:
•• **The ER chaperone MoLHS1 is involved in asexual development and rice infection by the blast fungus *Magnaporthe oryzae***. *Plant Cell* 2009, **21**:681-695.
This study established the important role for secreted proteins during biotrophic invasion of rice, and showed that blast effectors enter the normal ER-mediated secretion system for secretion and accumulation in BICs. *M. oryzae* mutants lacking the *LHS1* gene, which was identified based on sequence homology to *Saccharomyces cerevisiae Lhs1p* (luminal heat shock protein seventy), were severely impaired in biotrophic invasion of epidermal cells, in *AVR-Pita1*-mediated accumulation of GFP in BICs, and in induction of the *Pita*-mediated hypersensitive response. The mutants were defective in sporulation, but they showed only a minor impairment in growth on artificial culture media. This paper presents a comprehensive analysis of the unfolded protein response in *M. oryzae*.

24. Gilbert MJ, Thornton CR, Wakley GE, Talbot NJ: **A P-type ATPase required for rice blast disease and induction of host resistance**. *Nature* 2006, **440**:535-539.

25. Li W, Wang B, Wu J, Lu G, Hu Y, Zhang X, Zhang Z, Zhao Q,
•• Feng Q, Zhang H *et al.*: **The *Magnaporthe oryzae* avirulence gene *AvrPiz-t* encodes a predicted secreted protein that triggers the immunity in rice mediated by the blast resistance gene *Piz-t***. *Mol Plant–Microbe Interact* 2009, **22**:411-420.
This study reports cloning and characterization of *AvrPiz-t*, which corresponds to the rice *Piz-t* resistance gene [47] and provides a second blast *AVR* and *R* gene pair. AvrPiz-t suppresses BAX-mediated programed cell death in tobacco leaves, providing evidence that this effector functions in suppressing plant immunity. *Oryza* isolates lacking *AvrPiz-t* AVR function had either a single nonsynonymous nucleotide substitution or insertion of a transposable element in essential promoter sequences.

26. Dean RA, Talbot NJ, Ebbole DJ, Farman ML, Mitchell TK, Orbach MJ, Thon M, Kulkarni R, Xu J-R, Pan H *et al.*: **The genome sequence of the rice blast fungus *Magnaporthe grisea***. *Nature* 2005, **434**:980-986.

27. Yoshida K, Saitoh H, Fujisawa S, Kanzaki H, Matsumura H,
•• Yoshida K, Tosa Y, Chuma I, Takano Y, Win J *et al.*: **Association genetics reveals three novel avirulence genes from the rice blast fungal pathogen *Magnaporthe oryzae***. *Plant Cell* 2009, **21**:1573-1591.
This elegant study demonstrated the value of fungal genome resequencing and association genetics to clone 3 blast *AVR* genes. This paper is significant in several respects. First, in an attempt to identify *AVR* genes among the predicted secreted protein (pex) genes from the genome of sequenced laboratory strain 70-15, they showed that 78% of the 1032 pex genes tested showed no nucleotide polymorphisms in 46 diverse *Oryza* field isolates, consistent with recent dispersal of the pathogen. EcoTilling experiments used to detect nucleotide polymorphisms and PCR-analysis used to identify gain/loss alleles identified the control *AVR-Pita1* gene as highly polymorphic, and polymorphisms associated with *AVR-Pita1*'s AVR activity would have identified this gene. Therefore, the techniques were working but the targeted *AVR* genes were apparently not present in strain 70-15. Next, they resequenced Japanese rice isolate Ina168 (known to contain 9 *AVR* genes) and identified 1.68 Mb of Ina168 sequence that failed to align with the 70-15 genome. Associations between presence/absence polymorphisms and AVR activities led to identification of *AVR-Pia*, *AVR-Pii and AVR-Pik/km/kp* (accounting for 5 previously identified AVR specificities) among the 316 additional pex genes identified from Ina168. This study confirmed that gain/loss mutation is a major mechanism in blast *AVR* gene evolution. Therefore, resequencing and association genetics should be considered for cloning additional *AVR* genes. *AVR-Pia* was cloned independently in [28••].

28. Miki S, Kotaro M, Hideki K, Keisuke O, Taketo A, Nobuko Y,
•• Satoru F, Junko S, Kazuyuki H, Yoshikatsu F *et al.*: **Molecular cloning and characterization of the *AVR-Pia* locus from a Japanese field isolate of *Magnaporthe oryzae***. *Mol Plant Pathol* 2009, **10**:361-374.
Using rice isolate Ina168, *AVR-Pia* was cloned by isolation of DNA that had been deleted in a spontaneous mutant that had lost AVR activity toward a rice variety containing the *Pia* resistance gene. This study again highlights the general location of blast *AVR* genes in repeat-rich genomic regions, leading to frequent spontaneous deletions. *AVR-Pia* was cloned independently in [27••].

29. Ellis JG, Rafiqi M, Gan P, Chakrabarti A, Dodds PN: **Recent progress in discovery and functional analysis of effector proteins of fungal and oomycete plant pathogens**. *Curr Opin Plant Biol* 2009, **12**:399-405.

30. Hogenhout SA, Van der Hoorn RAL, Terauchi R, Kamoun S: **Emerging concepts in effector biology of plant-associated organisms**. *Mol Plant–Microbe Interact* 2009, **22**:115-122.

31. Stergiopoulos I, de Wit PJGM: **Fungal effector proteins**. *Annu Rev Phytopathol* 2009, **47**:233-263.

32. Jia Y, McAdams SA, Bryan GT, Hershey HP, Valent B: **Direct interaction of resistance gene and avirulence gene products confers rice blast resistance**. *EMBO J* 2000, **19**:4004-4014.

33. Ashikawa I, Hayashi N, Yamane H, Kanamori H, Wu J,
• Matsumoto T, Ono K, Yano M: **Two adjacent nucleotide-binding site-leucine-rich repeat class genes are required to confer *Pikm*-specific rice blast resistance**. *Genetics* 2008, **180**:2267-2276.
Rice resistance gene *Pikm* requires two adjacent NBS-LRR genes for activity.

34. Ma J-H, Wang L, Feng S-J, Lin F, Xiao Y, Pan Q-H: **Identification and fine mapping of *AvrPi15*, a novel avirulence gene of *Magnaporthe grisea***. *TAG Theor Appl Genet* 2006, **113**:875-883.

35. Kang S, Lebrun MH, Farrall L, Valent B: **Gain of virulence caused by insertion of a Pot3 transposon in a *Magnaporthe grisea* avirulence gene**. *Mol Plant–Microbe Interact* 2001, **14**:671-674.

36. Zhou E, Jia Y, Singh P, Correll JC, Lee FN: **Instability of the *Magnaporthe oryzae* avirulence gene *AVR-Pita* alters virulence**. *Fungal Genet Biol* 2007, **44**:1024-1034.

37. Fudal I, Böhnert HU, Tharreau D, Lebrun M-H: **Transposition of MINE, a composite retrotransposon, in the avirulence gene ACE1 of the rice blast fungus *Magnaporthe grisea***. *Fungal Genet Biol* 2005, **42**:761-772.

38. Farman ML, Eto Y, Nakao T, Tosa Y, Nakayashiki H, Mayama S, Leong SA: **Analysis of the structure of the *AVR1-CO39* avirulence locus in virulent rice-infecting isolates of *Magnaporthe grisea***. *Mol Plant–Microbe Interact* 2002, **15**:6-16.

39. Leong SA: **The ins and outs of host recognition of *Magnaporthe oryzae***. In *Genomics of Disease.* Edited by Gustafson JP, Taylor JW, Stacey G. Springer; 2008:199-216.

40. Tosa Y, Osue J, Eto Y, Oh HS, Nakayashiki H, Mayama S, Leong SA: **Evolution of an avirulence gene, *AVR1-CO39*, concomitant with the evolution and differentiation of *Magnaporthe oryzae***. *Mol Plant–Microbe Interact* 2005, **18**:1148-1160.

41. Tosa Y, Tamba H, Tanaka K, Mayama S: **Genetic analysis of host species specificity of *Magnaporthe oryzae* isolates from rice and wheat**. *Phytopathology* 2006, **96**:480-484.

42. • Faivre-Rampant O, Thomas J, Allegre M, Morel JB, Tharreau D, Notteghem JL, Lebrun MH, Schaffrath U, Piffanelli P: **Characterization of the model system rice-*Magnaporthe* for the study of nonhost resistance in cereals**. *New Phytol* 2008, **180**:899-910.
Together with [44•], this study initiates cytological and molecular analyses of *M. oryzae* strains adapted to wheat. Nonhost interactions involving a *M. oryzae* wheat isolate and a *M. grisea* crabgrass isolate were compared to host interactions with a *M. oryzae* rice isolate on three rice genotypes.

43. Heath MC, Valent B, Howard RJ, Chumley FG: **Interactions of two strains of *Magnaporthe grisea* with rice, goosegrass, and weeping lovegrass**. *Can J Bot* 1990, **68**:1627-1637.

44. • Tufan HA, McGrann GRD, Magusin A, Morel JB, Miche L, Boyd LA: **Wheat blast: histopathology and transcriptome reprogramming in response to adapted and nonadapted *Magnaporthe* isolates**. *New Phytol* 2009, **184**:473-484.
Together with [42•], this study initiates cytological and molecular analyses of the wheat blast fungus, in this case, during infection of wheat. Wheat responses to *M. oryzae* wheat isolates were compared to wheat responses to a nonhost *M. grisea* crabgrass isolate.

45. •• Soanes DM, Alam I, Cornell M, Wong HM, Hedeler C, Paton NW, Rattray M, Hubbard SJ, Oliver SG, Talbot NJ: **Comparative genome analysis of filamentous fungi reveals gene family expansions associated with fungal pathogenesis**. *PLoS ONE* 2008, **3**:e2300.
Comparative genome analysis was used to explore genetic features that differentiate plant pathogens from nonpathogens. Predicted proteins from genomes of 36 species of fungi and oomycetes, including 7 plant pathogens, were clustered into potential orthologous groups. Analysis of the species distribution of cluster members revealed filamentous fungal-specific proteins and plant pathogen-specific proteins. Comparison of gene inventories in pathogenic and nonpathogenic fungi identified gene families that are expanded in the plant pathogens, and thus are likely to be associated with pathogenic processes. No universal pathogenicity factors, completely specific to plant pathogen species, were identified. Protein families were identified that are more common in the secretomes of plant pathogens than nonpathogens. Among them, Kelch domain-containing proteins are implicated in cytoskeletal rearrangement and cell morphology. *M. oryzae* has four of these proteins, which may play a role in modifying rice cytoskeletal organization.

46. •• Choi J, Park J, Kim D, Jung K, Kang S, Lee Y-H: **Fungal Secretome Database: integrated platform for annotation of fungal secretomes**. *BMC Genomics* 2010, **11**:105.
The Fungal Secretome Database (FSD; http://fsd.snu.ac.kr) is an automated pipeline for predicting secreted proteins using a three-layer hierarchical rule based on nine prediction programs. This pipeline tool was used to predict putative secreted proteins from 158 fungal/oomycete genomes, and it is available at a user-friendly interface for use on new fungal genomes. The authors report that *M. oryzae* contains 2470 secreted proteins out of 11 069 total predicted proteins (22.3%), compared to related saprobe *Neurospora crassa* with 1666 secreted proteins out of 9842 total (16.9%). In general, plant pathogens have more putative secreted proteins than nonpathogens.

47. Zhou B, Qu S, Liu G, Dolan M, Sakai H, Lu G, Bellizzi M, Wang G-L: **The eight amino-acid differences within three leucine-rich repeats between *Pi2* and *Piz-t* resistance proteins determine the resistance specificity to *Magnaporthe grisea***. *Mol Plant–Microbe Interact* 2006, **19**:1216-1228.

48. Wang L, Xu X, Lin F, Pan Q: **Characterization of rice blast resistance genes in the *Pik* cluster and fine mapping of the *Pik-p* locus**. *Phytopathology* 2009, **99**:900-905.