Guo-Liang Wang
Barbara Valent
Editors

Advances in Genetics, Genomics and Control of Rice Blast Disease

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Front Cover: Neck blast. Photo by Barbara Valent from the field trip at the 3rd International Rice Blast Conference in Japan, 2003.
Back Cover: Projected confocal image of *Magnaporthe oryzae* invading rice sheath epidermal cells. The fungus expresses cytoplasmic EYFP (enhanced yellow fluorescent protein; pseudocolored blue) and nuclear-localizing mRFP (monomeric red fluorescent protein) at 36 hour post inoculation. The emboss effect was added using PowerPoint. Image from Chang Hyun Khang and Barbara Valent.

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Preface

Rice blast, caused by the fungus *Magnaporthe oryzae*, is one of the first recorded diseases of rice. It was known as rice fever disease in China as early as 1637 and was reported as *Imochi-byo* in Japan in 1704. As the rice production expanded through Asia, Latin America, and Africa over the last few centuries, the disease followed and is now found in over 85 countries worldwide. *M. oryzae* attacks all parts of the rice plant causing losses upwards of hundreds of millions of tons of rice grain annually. Such losses have lead to rice shortages in many developing countries in recent years, making effective control of this devastating disease imperative for global food security and social well-being.

Fortunately, the genomes of both rice and *M. oryzae* were completely sequenced within the last five years. Due to unrestrained public availability of the genome sequences, genetic stocks, and improved research tools, this pathosystem has become the premier model for understanding the molecular basis of plant-fungal interactions. Since the 3rd International Rice Blast Conference (IRBC) held on September 11–14, 2002 in Tsukuba, Japan, significant advancements have emerged in our understanding of this disease and technologies available for analysis. To review the recent progress and discuss the future of rice blast research, the 4th IRBC was convened on October 10–14, 2007 in Changsha, the capital of Hunan province, China.

The premise for having the conference in China is threefold. First, China is the largest rice producing country in the world with rice being the nutritional staple for over 60% of its 1.3 billion people. Sustainable rice production is essential for the economic and social stability of this country. Second, rice blast continues to cause significant yield losses in China, with many newly-bred rice varieties losing their genetic resistance within years of being released. Yield losses in a few elite hybrid rice lines were reported to be as high as 80–90% in recent years in hard hit regions. Finally, Chinese scientists began actively working on rice blast about 50 years ago. In the time since, they have made considerable achievements in selecting blast resistant germplasm for breeding programs, monitoring rice blast populations and epidemics, and developing effective cultural and chemical control protocols. Their recent progress in the molecular analysis of the defense response in rice and conversely pathogenicity in *M. oryzae* is laudable. China now has arguably the largest cohort of scientists in the world focusing on this disease. Therefore, the

proposal to hold the 4th IBRC in China was well received and fully supported by the Rice Blast Executive Committee.

The conference was successfully held in the new, well-appointed Vaya International Hotel. More than 250 attendees from 20 countries convened, making it the largest IRBC to date. There were 78 oral presentations and 64 poster presentations. Of special note, many postdoctoral fellows and graduate students, representing the future leaders of this field, attended the conference. A unique and memorable event was the award ceremony at the closing banquet in West Lake Restaurant. Distinguished Research Awards were presented to ten scientists who devoted their entire career to rice blast research. Eight of these distinguished scientists were able to attend the conference and accepted the recognition plaque prepared by the Rice Blast Executive Committee and the 4th IRBC Organizing Committee. The conference concluded with dramatic traditional Chinese dances and performances. By all accounts, the 4th IRBC was a success and once again united the international community of scientists studying this ancient and devastating disease of rice. We now look forward to hearing the next major advances and insights shared when rice blast research community assembles in Arkansas, USA in 2010.

Columbus, OH, USA Guo-Liang Wang

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Awardees of Distinguished Research Award on Rice Blast at the 4th IRBC

Professor FLEET LEE

Professor Lee, a faculty member at the University of Arkansas, first began working on blast in 1979, and has since received many awards and recognitions. He established the varietal blast screening system that allowed the Arkansas Cultivar Development Team to release many successful rice cultivars to farmers. Dr. Lee is described by team members as a "pathologist's pathologist", who makes careful field observations and then translates them into valuable information for farmers and agricultural scientists. He has been dedicated to understanding field resistance to blast, and through this dedication, he has optimized flood irrigation practices to help farmers to succeed in producing rice even when resistance genes have failed.

Professor LI, DEBAO

Professor Li is one of the pioneer molecular plant pathologists in China. He served as a deputy governor for Zhejiang province from 1983 to 1993. Professor Li helped to establish the Institute of Biotechnology at Zhejiang University, and he fostered and promoted rice blast research there. His latest contribution is to organize the Chinese rice blast researchers and initiate the biannual national rice blast conference in China. Thus, Professor Li has played a major role in promoting rice blast research throughout China.

Professor LING, ZHONGZHUAN

Professor Ling has worked with rice blast for over 45 years at the Institute of Crop Sciences, in the Chinese Academy of Agricultural Sciences. He has published over 80 research articles and four books. The neo-isogenic differential lines developed by Professor Ling have been widely used in China and in other countries. During his productive career, Professor Ling received 8 national and institutional awards for his contribution to rice blast research. Many highly resistant germplasms were identified by Professor Ling and used for breeding cultivars popular in northern

China. He also made contributions in establishing close collaboration of rice blast research between Chinese and Japanese scientists.

Professor LUO, KUAN

Professor Luo is a local hero from Changsha. He studied rice blast for 50 years at the Hunan Agriculture University. During this time, he published more than 70 research articles on physiological races and virulence of rice blast isolates from Hunan and nearby provinces, and on disease resistance and control. He is the recipient of a first class Technology Improvement Award from the Chinese Ministry of Agriculture and four Science and Technology Progress Awards from Hunan provincial government.

Dr. HAJIME KATO

Dr. Hajime Kato began working on rice blast in 1958, and worked for more than 40 years before retiring. Since retirement, he continues his involvement with rice blast. During his distinguished career, Dr. Kato focused on the epidemiology of blast disease on rice and millets, and on the inoculum source problem for panicle blast. Dr. Kato was one of the pioneers in characterizing the sexual state of the fungus, and he has been dedicated to understanding the relationship among blast isolates from different plant species. Dr. Kato's discovery that the fungus produces microconidia with some unknown role showed blast researchers how much there still is to learn about this important disease.

Dr. JEAN-LOUP NOTTEGHEM

One of Dr. Notteghem's major contributions is the building and maintenance of one of the best historical, world-wide collections of field isolates of the rice blast fungus. In this process, Dr. Notteghem discovered the most famous fungal strain, GUY11. Dr. Notteghem's main research theme has been searching for and characterizing upland rice cultivars with high levels of partial resistance and naturally pyramided resistance genes. This resulted in availability of durably-resistant varieties to breeders, such as Moroberekan and IRAT13. Dr. Notteghem was a pioneer in mixing classical plant pathology and genetics of host-fungus interaction, and in adding in molecular biology. He now leads a team that uses all the modern genomic tools available to study rice resistance and the rice blast pathogen.

Dr. ANNE SITARAMA PRABHU

Dr. Prabhu began working on blast in Brazil in 1973. He was a pioneer in establishing the first coordinated rice blast program in this large and diverse country. He established a national rice blast nursery, which resulted in the first blast resistant variety in the field in 1986. Dr. Prabhu received many awards and recognitions,

including the prestigious highest award given to a Brazilian scientist working in the national research system. He has collaborated with scientists internationally. However, the unique upland rice that is best adapted in Brazil had to be developed in Brazil. Dr. Prabhu's research resulted in valuable quasi-isogenic lines containing 13 resistance genes. Since having resistant rice is not enough for farmers, another major contribution of his was to develop disease management strategies for farmers "to learn to live with rice blast disease".

Professor PENG, SHAOQUI

Professor Peng graduated from Yangzhou Agriculture University and retired from the Institute of Plant Protection, Hunan Academy of Agricultural Sciences few years ago. Dr. Peng has studied plant pathology, including rice blast, for 50 years. He has undertaken three projects in the Natural (National) Science Foundation of China on "Durable Resistance to Rice Blast". He has taken part in more than 20 national or provincial programs. He has achieved impressive results applying geo-phytopathology to his durable resistance studies on rice blast and plant protection in general. He has been especially active in proposing and promoting study of durable blast resistance at all times within China.

Dr. HIROSHI YAEGASHI

Dr. Yaegashi graduated from Chiba University in Japan in 1965, and he worked on rice blast until his retirement two years ago. Dr. Yaegashi was one of the pioneers in characterizing the sexual state of the rice blast fungus. Many blast researchers have often referenced his famous publication, Yaegashi and Udagawa, 1978, on "The taxonomical identity of the perfect state of *Pyricularia grisea* and its allies." Dr. Yaegashi published a paper in 1981 that showed that a finger millet pathogen contains avirulence genes that correspond to resistance genes in rice. Thus, Dr. Yaegashi led the way in studying the genetics of host specificity in the pathogen.

Professor SHEN, YING

Professor Shen's career with rice blast spanned over four decades in two provinces, Sichuan and Zhejiang. She contributed significantly to understanding genetic diversity, population structure, and geographic distribution of *Magnaporthe oryzae* in China and other Asian countries. Professor Shen also was involved in evaluation and utilization of rice germplasm resistant to blast and other major rice diseases. She has authored over 110 papers and received numerous awards from the central and provincial governments. Professor Shen has been actively involved in many international collaborative projects with scientists in US, Japan, IRRI and Europe.

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Current Status and Future Prospects for Research on Blast Resistance in Rice (*Oryza sativa* L.)

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Abstract Rice is the most important food security crop and staple food of half of the world population. Major increases in rice production occurred during the past four decades of last century as a result of adoption of green revolution technology. Demand for rice continues to increase as a result of population increase and improvement in living standards particularly in Africa and Latin America. However, rate of increase of rice production has slowed down. It is estimated that we will have to produce 30% more rice in 2030. For this purpose we need rice varieties with higher yield potential and greater yield stability. Breakdown of blast resistance is the major cause of yield instability in several rice growing areas. Efforts are underway to develop rice varieties with durable blast resistance. More than 40 major genes as well as QTL for blast resistance have been identified. Monogenic resistance to blast is less stable but varieties with pyramided monogenes or QTLs are durably resistant. Rice research should focus on identifying more durably resistant genes, tagging of these genes with molecular markers and pyramiding these genes or QTLs through molecular marker-aided selection. Candidate gene identification through rice functional genomics has great potential for developing more durably resistant varieties.

Keywords Monogenes · QTL · Resistance breakdown · Durable resistance · Gene nomenclature

1 Introduction

Rice is the world's most important food crop and a primary source of food for more than half of the world's population. More than 90% of the world's rice is grown and consumed in Asia where 60% of the world's people live. Rice accounts for

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35–75% of the calories consumed by more than 3.0 billion Asians. Major increases in rice production occurred during last four decades due to the adoption of green revolution technology. However, the rate of growth of rice production has slowed down. Whereas rice production increased at the annual growth rate of 2.49% during 1970–1990, the annual growth rate was 1.70% during 1990–2000 and only 1.21% during 2000–2006. World rice stocks are at the lowest level since 1974 and price of rice is showing upward trend in the domestic and international markets.

The population of rice consumers is continuing to increase and demand for rice is also going up due to improved living standards particularly in Africa. According to various estimates we will have to produce 30% more rice by 2030. To meet this challenge we need rice varieties with higher yield potential and greater yield stability. Although yield potential of rice is 10 tons per hectare, farmers on the average harvest about 5 tons per hectare from irrigated lands. This yield gap is due to the losses caused by biotic and abiotic stresses. Among the biotic stresses blast disease is most important.

2 Rice Blast Problem and Yield Loss

Blast disease affects rice production in all rice growing regions. However, the yield losses are most serious in temperate regions and upland condition. Stresses caused by low temperature in temperate areas and moisture stress under upland conditions increase the vulnerability of the crop to blast disease attack. During the last 30 years, there were three outbreaks of blast in China; 1982–1985, 1992–1994, and 2001–2005. The average blast infected area was more than 3.8 million hectares in 1982–1985 with yield losses of several million tons (Sun et al. 1999). In 1993, yield loss of 1.1 million tons was recorded in Southern China alone. In recent years 5.7 million hectares of rice fields were affected by the blast disease.

Blast disease is a major constraint in certain agro-ecological regions of India. Eastern India has the highest occurrence of blast followed by North and South. Sometimes yield losses reach as high as 50% in upland conditions (Widawsky and O'Toole 1990). Production oriented survey is organized by Directorate of Rice Research, Hyderabad every year to document constraints to rice production in various parts of the country. Multidisciplinary teams of scientists survey the crop in the 125 districts of the country. About 15% of the districts surveyed during 2001–2005 had severe blast disease. Eastern India had the highest incidence of blast over 12-year period. Hill districts of Himachal Pradesh, Uttaranchall, and Jammu and Kashmir with cool climate generally have higher incidence of blast (Variar 2007).

The cool temperate climate of Japan is highly conducive to the multiplication and spread of the blast fungus during the rice growing season. Even though breeding for blast resistance has been emphasized in Japan since the beginning of last century and a number of genes for blast resistance have been introduced in Japanese varieties. Frequent breakdown of resistance results in significant yield losses ranging from 20–100% in some areas. In 1993 exceptionally cool weather resulted in blast epidemic.

Nation wide yield loss that year was 45.2% due to blast damage and sterility caused by low temperature and Japan was forced to import large quantities of rice from abroad.

South Korea with temperate climate is another country where blast disease causes yield losses every year. During the blast epidemic of 1984–1985 almost 20% of the rice area was seriously blasted.

In Indonesia 1.1 million hectares are planted to upland rice where blast is a serious problem. Upland rice varieties become susceptible within one to two years of their cultivation. Yield losses will be as high as 100% during certain years. In 2004 several hundred hectares of upland rice in Lampung province could not be harvested due to blast damage and yield losses up to 70% were recorded in upland areas of West Java (Sobrizal et al. 2007).

3 Genetics of Resistance

Studies on inheritance of resistance were first reported in 1922 by Sasaki followed by Takahashi (1965) and Yamasaki and Kiyosawa (1966). Up to 1960 the inheritance was studied without sufficient knowledge of the pathogenic specialization of the causal fungus, and it was quite difficult to obtain universal understanding of the subject. Systematic studies were undertaken only after Goto (1965) established the differential system for blast fungus races in Japan.

Kiyosawa and his colleagues used seven Japanese strains of blast fungus for investigating the inheritance of resistance and identified 13 genes for resistance (Kiyosawa 1981). These were designated as *Pi-a*, *Pi-i*, $Pi\text{-}k^s$, *Pi-k*, *Pi-z*, *Pi-ta*, $Pi\text{-}ta^2$, $Pi\text{-}z^t$ $Pi\text{-}k^p$, $Pi\text{-}k^m$, $Pi\text{-}k^n$, *Pi-b, and Pi-t*. With the establishment of Rice Genetic Cooperative in 1985, rules for gene symbolization in rice were standardized and it was decided to assign gene symbols derived from names which describe the character modifications. The genes that are non-allelic but are indistinguishable from each other phenotypically, are designated by the same base letter but are differentiated by a number or a letter subscript separated by a hyphen (Kinoshita 1986). Since then several genes for resistance have been designated by base letter followed by a number subscript starting with *Pi-1*, *Pi-2* (Mackill and Bonman 1992) and so on up to *Pi-44*.

During the third International Rice Genetics Symposium held at Manila on the October 16–19, 1995, it was decided to remove the hyphen between the base letter and subscript. Henceforth, the gene symbols have been written as *Pia*, *Pita*, *Pik*, *Pi1*, *Pi2* etc.

During the past 25 years great advances have been made in studying the genetics of resistance to the blast disease. Following conventional genetic analysis of identified donors with resistance, availability of pure isolates of the blast pathogen and use of advanced molecular analysis techniques, about 60 genes for resistance have been identified. These have been designated as *Pi1*, *Pi2*, *Pi3*, *Pi4*, *Pi5*, *Pi6*, *Pi7*, *Pi9*, *Pi10* and *Pi11* (Causse et al. 1994, Wang et al. 1994), *Pia*, *Pib*, *Pik*, *Pit*, *Pita*, $Pita^2$,

Pi12, *Pi17*, *Pi18*, *Pi19*, *Pi20*, *Pi23*, *Pi57*, *Pi62* (Nagato and Yoshimura 1998), *Pii* and *Pi15* (Pan et al. 2003), *pi21* (Fukuoka and Okuno 2001), *Pi25* (Yang et al. 2001), *Pi27* (Zhu et al. 2004), *Pi24*, *Pi25*, *Pi26*, *Pi27*, *Pi28*, *Pi29*, *Pi30*, *Pi31* and *Pi32* (Sallaud et al. 2003), *Pi33* (Berruyer et al. 2003), *Pish* (Fukuta et al. 2004), *Pi35* (Nguyen et al. 2006), *Pi36* (Liu et al. 2005), *Pi37* (Chen et al. 2005), *Pi38* (Gowda et al. 2006), *Pi39* (Liu et al. 2007) and *Pi40* (Jeung et al. 2007).

With the advent of molecular markers various genes for blast resistance have been located to specific chromosomes. For example *Pi2* and *Pi4* have been located on chromosome 6 and 12 respectively via linkage with RFLP markers (Yu et al. 1991). Near-isogenic lines (NILs) were used for genetic mapping by dissecting complex genotypes and evaluating the effect of individual genes in a common genetic background. By using a pair of NILs, *Pi1* was located on chromosome 1 and *Pita* on chromosme 12 (Yu et al. 1996). Near-isogenic lines with single genes for resistance have been developed in the genetic backgrounds of the indica variety CO39, Japonica variety Lijiangxintuanheigu (LTH) and a universal susceptible line US2. These isogenic lines developed at IRRI through a collaborative project between IRRI and Japan International Research Center for Agricultural Sciences (JIRCAS) (Kobayashi et al. 2007) are very useful for identification of the blast races, identifying genes for blast resistance in donors and for monitoring the shift in blast races.

A mapping population developed at IRRI from a cross between IR64 and Azucena has been used world wide for mapping genes for blast resistance. Sallaud et al. (2003) identified nine genes derived from IR64. Recombinant inbred lines (RILs) have been used for mapping four major QTLs for the blast resistance via linkage to RFLP markers (Tabien et al. 2000). Simple sequence repeats (SSR) markers have been used for fine mapping the Pik^h gene which confers resistance to the blast races of Northwestern Himalayan region of India (Sharma et al. 2005). The genes *Pi3* and *Pi5* were found to be located on chromosome 9 via their linkage to AFLP markers (Jeon et al. 2003). Single nucleotide polymorphism (SNP) markers have been employed for fine mapping the *Piz* locus (Hayashi et al. 2004). The resistance gene, *Pi39* from Chinese variety, Q15 has been fine mapped on chromosome 12 (Liu et al. 2007). More recently a novel gene, *Pi40* from the breeding line IR65482-4-136-2-2 with strong resistance to Korean and Philippine blast isolates was mapped on the short arm of chromosome 6 (Jeung et al. 2007).

Advances in molecular genetics and completion of the genome sequence of rice paved the way for cloning and characterization of seven major genes for blast resistance. These are *Pib* (Wang et al. 1999), *Pita* (Bryan et al. 2000), *Pi9* (Qu et al. 2006), *Pi2*, and Piz^t (Zhou et al. 2006), *Pid2* (Chen et al. 2006) and *Pi36* (Liu et al. 2007). All the cloned resistance genes belong to the NBS-LRR class of R genes except *Pid2* which encodes a receptor-like kinase protein with a predicted extra-cellular domain of bulb type manose specific binding lectin (B-lectin) and an intracellular serine threonine kinase domain. The several R genes for the blast resistance, the genes located at *Piz*, *Pik*, and *Pita* loci on chromosome 6, 11, and 12 respectively are clustered with a similar allelic expression for resistance (Monosi et al. 2004).

4 Breeding for Resistance to Blast

Efforts have been made to develop rice varieties with blast resistance since the beginning of rice improvement programs particularly in Japan. Major genes for blast resistance have been utilized in many breeding programs. These genes are easier to move from one varietal background to the other. Thus *Pib*, *Pita*, *Pia*, *Pi1*, Pik^h, *Pi2* and *Pi4* have been introduced into rice varieties through conventional breeding programs. In recent years MAS has been employed for transferring *Pita* (Rybka et al. 1997), *Pi1*, *Pi9* (Du et al. 2007), *Piz* (Conaway-Bormans et al. 2003), *Pi35* (Nguyen et al. 2006) and *Pi37* (Chen et al. 2005) to new varieties.

Insufficient durability of monogenic blast resistance is a serious problem especially in upland rice and temperate irrigated rice. Several blast resistant varieties are released by rice breeding programs and adopted by farmers only to loose their resistance within a few years because of the adaptation of the blast pathogen. The loss of resistance has been most striking and well documented (Kiyosawa 1982; Koizumi 2007).

Several breeding strategies have been suggested for prolonging the durability of resistance. These include; (1) multiline varieties, (2) varietal mixtures, (3) pyramiding of major genes for resistance and (4) breeding for quantitative or partial resistance based on minor genes or QTLs. Multiline approach was first suggested by Borlaug (1959) for developing wheat varieties with resistance to rust and implemented by Browning and Frey (1969) for control of crown rust of Oats. This approach has not been tried by rice breeders. The durability of resistance of multiline varieties would depend on the rate at which the blast races develop, the number of component lines in a mixture and the extent of area planted to multiline varieties. Another strategy for slowing the development of the blast races is the varietal mixtures consisting of 80–90% resistant plants and 10–20% susceptible plants of similar varietal background. Varietal mixtures are easy to introduce but their agronomic uniformity have to be assured. A variation of this approach was tried in Yunan province of Southeast China. Highly susceptible glutinous plants were mixed planted with non-glutinous hybrid indica rice. The mixtures reduced blast development on glutinous rice and were deployed on about 1 million hectare in 2004 (Leung et al. 2003; Zhu et al. 2005). For the varietal mixtures interplanting 1 row of taller glutinous rice varieties for every 4–6 rows of hybrid indica rice was planted. However, the inter-planting patterns have been decided empirically while reduced leaf wetness is supposed to be a substantial contributor to panicle blast control in mixtures (Zhu et al. 2005).

The quantitative or polygenic resistance is governed by a large number of genes also called QTL each with a small contribution to resistance. The level of resistance is generally not very high. So it does not exert strong selection pressure on the pathogen. The gene for gene relationship does not exist between the QTL for resistance and the pathogenic races (Robinson 1980). However, there are practical difficulties in incorporating QTLs in high yielding agronomically acceptable background. Parents with QTLs are generally land races with poor agronomic traits. In the process of selecting plants with better agronomic traits involving such traits,

not all QTL are transferred. This problem can be overcome by tagging the QTLs with molecular markers and accumulating the QTLs through MAS. Another problem is that many of the techniques employed in screening segregating progenies usually favor the selection of genotypes with high level of resistance conditioned by major genes.

In the rice breeding program at IRRI the emphasis was on the incorporation of quantitative resistance. For this purpose segregating populations were evaluated in the blast nursery where numerous races of the blast pathogen were present. Screening started with F_2 generation. F_2 populations were planted in the blast nursery. Seedlings with a high level of resistance and those that were highly susceptible were discarded. Only those with an intermediate score of 3–4 on a scale of 1–9, were selected and planted in F_3 nursery. Breeding lines were continuously screened in subsequent generations by planting in the blast nursery and only those with moderate level of resistance were advanced to the next generation. Thus through evaluation for the blast resistance for at least 6–7 generations in blast nursery lines with moderate level of resistance presumably governed by polygenes were selected (Khush and Virk 2005). Although we did not try to incorporate major genes intentionally, genetic analysis had shown that IR varieties also have a few major genes for resistance (Imbe et al. 2000; Ebron et al. 2004). Since we used diverse parents in our hybridization program and some of these parents have major genes, these genes were selected because of the presence of compatible races of blast pathogen in the blast nursery. Through this process of hybridization and selection major as well as polygenes were combined in most of the IR varieties. IR varieties have been grown widely under irrigated conditions and have not suffered any major damage from blast. Only exception is IR50 which has shown blast susceptibility at a few locations and obviously lacks QTLs for resistance. Both IR36 and IR50 have major genes *Pita* and *Pib* (Imbe et al. 2000). But IR36 has shown durable resistance. It was grown to almost 11 million hectares during 1980s and is still popular in several areas. The durable resistance of IR36 is expressed through a reduction in lesion size and number (Bonman et al. 1992).

One of the complications encountered in breeding for blast resistance is that selection is most easily made based on observation of leaf blast, while the infection of greatest economic importance occurs on the panicle and panicle neck node. Panicle blast resistance is difficult to measure because the occurrence of panicle infection is influenced by weather, and even small differences in maturity between lines can result in inaccurate assessment of their level of resistance. There is, however, a correlation between partial resistance to leaf blast and partial resistance to panicle blast with a few exceptional varieties being susceptible to leaf blast yet resistant to panicle blast (Bonman et al. 1989).

5 Future Prospects

For several years gene symbols for blast resistance were monitored by a committee established by Rice Genetics Cooperative. However, during recent years rules for assigning gene symbols have not been followed strictly by all authors. Note for

example gene symbols *Pitq*[5], *Pitq*[6] for genes from variety Teqing or *PiCO39*(t) from rice variety CO39 as well as *PiD1* and *PiD2* from rice variety Degu (cf. Table 1 in Koizumi 2007). These gene symbols are invalid. Another problem is that although more than 60 genes have been described, allelic relationships of many of them have not been investigated. Several of them are likely allelic and the actual number is probably much lower.

There is also some confusion regarding the distinction between major genes and QTLs. What are the criteria for distinguishing between the two? Some major genes for resistance have been referred as QTL by other authors. How do you assign gene symbols for QTLs distinct from major genes? Since rice blast workers meet on regular intervals a committee should be appointed for monitoring symbolization of genes for the blast resistance. Whenever, a new gene is discovered, the chairman of the committee should be consulted for assigning gene symbol. The committee should also encourage the studies on determination of allelic relationships of already described genes as well as new genes

Remarkable progress has been made in identifying and tagging of blast resistance genes with molecular markers. Almost all the genes identified to date are for leaf blast resistance. However, neck blast is more important. Therefore, efforts should be made to identify and tag genes for resistance to neck blast. Breeding for neck blast resistance will then become feasible.

Rice breeders now have a number of genes for resistance to work with. However, most of the breeding programs have emphasized monogenic resistance. Since many of the genes have now been tagged with molecular markers, it should be feasible to pyramid several genes into improved varieties. Useful life of such varieties would be considerable as exemplified by IR64. The variety IR64 has at least five genes for blast resistance and has shown durable resistance for the last 20 years.

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Part I
Pathogen Genetics, Genomics and Molecular Biology

The *PMK1* MAP Kinase Pathway and Infection-Related Morphogenesis

Shengli Ding, Xiaoying Zhou, Xinhua Zhao and Jin-Rong Xu

Abstract Appressorium formation is an essential step in the infection cycle of *Magnaporthe oryzae*. The fungus can recognized hydrophobic surface to initiate appressorium formation *via* the cAMP signaling pathway, but the differentiation of mature appressoria and invasive hyphae is regulated by the Mst11-Mst7-Pmk1 MAPK cascade. Mutants blocked in the Pmk1 pathway are nonpathogenic. Mst50 directly interacts with both Mst11 and Mst7 and functions as the adaptor protein for transducing upstream signals to the Pmk1 cascade. Both Mst50 and Mst11 have the Ras association domain and physically interact with Ras1 and Ras2, two Ras proteins in *M. oryzae*. Ras2 appears to play critical roles in the activation of both the cAMP-signaling and Pmk1 MAPK pathways. When Pmk1 is activated by Mst7, it may in turn activate multiple downstream transcription factors because Mst12 is only required for appressorial penetration. Genes regulated by *PMK1* have been identified by several approaches but only a few of them have been functionally characterized. It will be important to identify and characterize other transcription factors and infection-related genes regulated by the Pmk1 pathway.

Keywords Appressorium · cAMP signaling · Invasive growth · MAP kinase · Penetration.

1 Introduction

Rice blast disease caused by *Magnaporthe oryzae* is one of the most severe fungal diseases of rice throughout the world (Valent and Chumley 1991). The infection cycle of this pathogen begins with the attachment of conidia to rice plants. Germ tubes produced from conidia differentiate into specialized infection structures called appressoria. The fungus uses the turgor pressure generated in appressoria to penetrate the plant cuticle and cell wall. After penetration, the penetration peg differentiates into unbranched primary infectious hyphae, which in turn develop

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bulbous, lobed invasive hyphae in plant cells. As a hemibiotrophic pathogen, *M. oryzae* initially grows biotrophically in infected tissues (Kankanala et al., 2007). Eventually, lesions develop on rice plants and the fungus produces more conidia to reinitiate the infection cycle.

2 *PMK1* is Essential for Plant Infection

Although attachment is sufficient to stimulate conidium germination, the differentiation of appressoria at germ tube tips in *M. oryzae* is affected mainly by surface hydrophobicity. Surface recognition and the initiation of appressorium formation is regulated by the cAMP signaling pathway (Fang and Dean, 2000; Zhao et al., 2007). However, the formation of mature, melanized appressoria is regulated by the *PMK1* (Pathogenicity MAP Kinase 1) gene (Xu and Hamer, 1996). Deletion of *PMK1* blocks appressorium formation and plant infection. The *pmk1* deletion mutant fails to form appressoria on any artificial and plant surfaces tested, but it still recognizes hydrophobic surfaces and responds to extracellular cAMP and forms subapical swollen bodies that are irregular in shape, unmelanized, and smaller than normal appressoria. *PMK1* also is essential for invasive growth after penetration (Xu and Hamer, 1996). Further analyses indicate that *PMK1* is required for root infection in *M. oryzae* under laboratory conditions (Sesma and Osbourn, 2004), and it regulates the mobilization of storage carbohydrate and lipid reserves from conidia to appressoria (Thines et al., 2000).

PMK1 is homologous to *FUS3* and *KSS1* in *Saccharomyces cerevisiae*, which are two MAP kinase genes with partially overlapping functions in mating and filamentous growth. It can partially suppress the mating defect of a yeast *fus3 kss1* double mutant (Xu and Hamer, 1996). In *M. oryzae*, the *pmk1* mutant is female sterile but retains male fertility. *PMK1* is expressed and activated constitutively at a relatively low level in vegetative hyphae (Bruno et al., 2004; Zhao et al., 2005). In transformants expressing a *GFP-PMK1* fusion construct that can fully complement the *pmk1* mutant, GFP signals are weak in vegetative hyphae, conidiophores, mature conidia, and germ tubes. The expression of GFP-*PMK1* is increased in developing appressoria and GFP-Pmk1 fusion proteins localize to nuclei (Bruno et al., 2004). In invasive hyphae, GFP signals also are detected but not localized to nuclei.

3 The *MST11-MST7-PMK1* MAP Kinase Cascade is Well Conserved

A typical MAP kinase pathway consists of the MAP kinase (MAPK), MAP kinase kinase (MEK), and MEK kinase (MEKK) that are sequentially activated. *MST11* and *MST7* are the MEKK and MEK genes (Fig. 1), respectively, that function upstream from *PMK1* (Zhao et al., 2005). The *mst7* and *mst11* deletion mutants also are nonpathogenic and fail to form appressoria. Expression of the dominant active $MST7^{S212D\ T216E}$ allele rescues the defects of the *mst7* and *mst11* mutants in appressorium formation and phosphorylation of Pmk1, but has no obvious effects

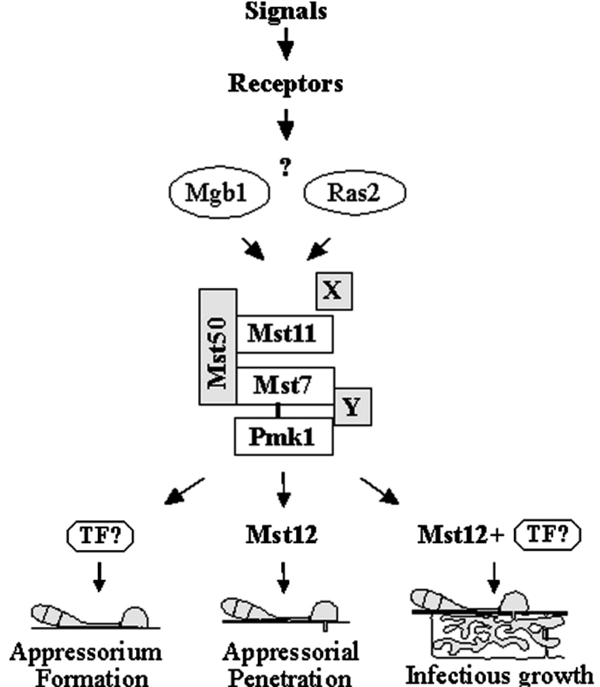

Fig. 1 The *PMK1* MAPK pathway. The Mst11-Mst7-Pmk1 cascade is well conserved and required for appressorium formation, penetration, and invasive growth. Mst50 functions as an adaptor by binding to Mst7, Mst11, Ras2, and Mgb1. Mst11 may be a convergence point for the upstream Mgb1 and Ras2 signaling. Other unidentified factors (X and Y) may be responsible for the activation of Mst11, the enhanced interaction of Mst7 with Pmk1, and the specificity of multiple signal inputs and outputs of the PMK1 pathway. Mst12 and other transcription factors (TFs) are regulated by Pmk1 to control different infection processes

on the *pmk1* mutant (Zhao et al., 2005). In addition to the C-terminal protein kinase domain, the Mst11 MEKK has one sterile alpha motif (SAM) and one Ras-association (RA) domain. Deletion analyses have revealed that the SAM domain is essential for the function of *MST11*. Expression of the $MST11^{\Delta SAM}$ allele fails to complement the *mst11* mutant. In contrast, the RA domain is dispensable for appressorium formation, although transformants with $MST11^{\Delta RA}$ tend to form multiple appressoria on branching germ tubes (Zhao et al., 2005).

In yeast two-hybrid assays, Mst11 weakly interacts with Mst7, but direct interaction of Pmk1 with Mst7 or Mst11 is not observed. The*in vivo* association of Mst7 with Pmk1 is detected by the co-immunoprecipitation (co-IP) and bimolecular fluorescence complementation (BiFC) assays during appressorium formation (Zhao and Xu, 2007). However, the Pmk1-Mst7 interaction is not detectable in vegetative hyphae, conidia, and germ tubes. Mst7 contains an N-terminal MAPK-docking site that is essential for the interaction of Mst7 with Pmk1. The corresponding docking region of *PMK1* also is required for the Pmk1-Mst7 interaction (Zhao and Xu, 2007). These observations indicate that the interaction between Mst7 and Pmk1 is mediated by the docking site.

4 Mst50 Functions as an Adaptor Protein for the *PMK1* Pathway

The *MST50* gene in *M. oryzae* is homologous to yeast *STE50*. Deletion of *MST50* also blocks appressorium formation and plant infection (Park et al., 2006). Expression of the dominant active *MST7* allele in the *mst50* mutant increases the level of Pmk1 phosphorylation and suppresses its defect in appressorium formation, indicating that Mst50 functions upstream from the Mst11-Mst7-Pmk1 cascade. In yeast two-hybrid assays, Mst50 directly interacts with both Mst7 and Mst11 but not Pmk1. The *in vivo* association of Mst50 with Mst7 and Mst11 has been determined by co-immunoprecipitation assays (Park et al., 2006). Mst50 also has one SAM and one RA domain. The SAM domain mediates the interaction of Mst50 with Mst11 and is essential for its function (Park et al., 2006; Zhao et al., 2005). The function of the RA domain is not clear, but Mst50 interacts with both Ras1 and Ras2, two Ras proteins in *M. oryzae*. Mst50 also interacts with the G-beta subunit Mgb1 (Nishimura et al., 2003) and MgCdc42 (homolog of yeast Cdc42) in yeast two-hybrid assays. Multiple upstream signals may converge on Mst50 for activating the downstream Pmk1 MAPK cascade.

5 The PAK Kinase Genes Are Dispensable for Appressorium Formation in *M. oryzae*

In yeast, the PAK kinase Ste20 plays an essential role in the pheromone response and filamentous growth pathways. In *M. oryzae*, *MST20* (the *STE20* homolog) is dispensable for *PMK1* activation or appressorium formation. The *mst20* deletion mutant has no obvious defects in growth and plant infection (Li et al., 2004). *CHM1*, a homolog of yeast *CLA4*, is the only other PAK kinase gene in *M. oryzae*. It is also dispensable for appressorium formation. The *chm1* mutant has pleiotropic defects in growth, conidiation, and plant infection. Although it still forms appressoria, the *chm1* mutant is defective in plant penetration. It is likely that neither *MST20* nor *CHM1* is essential for the activation of the Mst11-Mst7-Pmk1 cascade and appressorium formation, although the latter may be involved in various developmental and plant infection processes in *M. oryzae*. In *Ustilago maydis*, the Smu1 PAK kinase also is dispensable for the activation of Kpp4 MEKK, mating, and plant infection (Smith et al., 2004).

6 Ras2 May Function Upstream From the Pmk1 Mapk Pathway

The two Ras proteins, Ras1 and Ras2, both directly interact with Mst50 and Mst11 in yeast two-hybrid and co-IP assays (Park et al., 2006). While *RAS1* is dispensable for growth and appressorium formation, *RAS2* appears to be an essential gene in

M. oryzae. In the earlier version of automated annotation, five amino acid residues towards the N-terminus of *RAS2* were predicted incorrectly. Expression of a putative dominant negative allele ($RAS2^{S23N}$) in the wild-type strain has no obvious effect on vegetative growth and plant infection. When a dominant active *RAS2* allele ($RAS2^{G18V}$) is expressed in the wild-type strain, it stimulates appressorium formation on both hydrophobic and hydrophilic surfaces (Park et al., 2006). However, appressorium formation was not observed in transformants of the *pmk1* and *mst7* mutants expressing the $RAS2^{G18V}$ allele. Therefore, *RAS2* must function upstream from the *PMK1* MAPK cascade for regulating appressorium formation.

In the wild-type background, expression of the $RAS2^{G18V}$ allele also resulted in the formation of melanized appressorium-like structures on aerial hyphae and conidiophores. The level of intracellular cAMP was elevated in those transformants (Zhao and Xu, unpublished). Expression of the $RAS2^{G18V}$ allele has no obvious effect in the *cpkA* mutant, suggesting that *RAS2* may also function upstream from the cAMP signaling pathway. Constitutive activation of Ras2 may result in the overactivation of both the cAMP and *PMK1* MAPK pathways and allow the fungus to bypass the surface attachment and recognition signals for appressorium formation.

The switching between active GTP- and inactive GDP-bound forms of Ras proteins is controlled by RasGEF (guanine nucleotide exchange factors) and RasGAP (GTPase activating proteins). The *M. oryzae* genome contains four putative RasGEF genes (MGG_02419.5, MGG_00371.5, MGG_11785.5, MGG_00199.5) and four putative RasGAP genes (MGG_11425.5, MGG_08105.5, MGG_03846.5, MGG_03700.5). None of these genes has been functionally characterized, and it will be interesting to determine which ones function as the actual RasGEF and RasGAP for Ras2 in *M. oryzae*. In mammalian cells, receptor kinases may function upstream from Ras proteins. Unfortunately, similar to other sequenced filamentous ascomycetes, *M. oryzae* lacks any recognizable receptor protein kinase genes.

7 Trimeric G-Proteins

For the yeast mating pathway, the binding of pheromone to receptor results in the dissociation of Gpa1-GTP from the Gβγ subunits, which in turn activates the downstream MAP kinase cascade (Bardwell, 2004). Among three Gα genes in *M. oryzae*, only *MAGB* is involved in appressorium formation and plant infection. The *magB* deletion mutant exhibits reduced appressorium formation and virulence but is still pathogenic (Fang and Dean, 2000; Liu and Dean, 1997). Expression of a dominant active *MAGB* allele in the wild type stimulates appressorium formation on hydrophilic surfaces but reduces virulence on rice seedlings (Fang and Dean, 2000). While the Gγ gene has not been characterized, deletion of the Gβ gene *MGB1* blocks appressorium formation and plant infection (Nishimura et al., 2003). Unlike the *pmk1* mutant, the *mgb1* mutant forms fluffy colonies on oatmeal agar plates that produce abundant aerial hyphae but only few conidiophores and conidia. It has a reduced intracellular cAMP level. Exogenous cAMP induces

appressorium formation in the *mgb1* mutant. Several suppressor mutants isolated from the *mgb1* mutant exhibit improved conidiation, but are still nonpathogenic. Appressoria formed by these suppressor mutants are defective in plant penetration (Nishimura et al., 2003). Recently, a negative regulator of G-proteins, *RGS1*, has been shown to physically interact with all three G-alpha subunits in *M. oryzae* (Liu et al., 2007). The *rgs1* deletion mutant forms appressoria on hydrophilic surfaces and has elevated levels of intracellular cAMP.

Trimeric G-proteins are activated by G-protein-coupled receptors (GPCRs) that normally contain seven transmembrane domains. The genome of *M. oryzae* contains a large number of GPCR-like genes (Kulkarni et al., 2005). Twelve of them, including *PTH11*, form a subfamily and contain a fungal-specific, extracellular membrane-spanning domain (CFEM). *PTH11* is involved in surface recognition and pathogenesis in *M. oryzae* (DeZwaan et al., 1999). The *pth11* mutant is significantly reduced in appressorium formation and virulence. However, it is likely that *PTH11* functions through the cAMP signaling pathway because exogenous cAMP can suppress the defect of the *pth11* mutant in appressorium formation and plant infection.

8 Downstream Transcription Factors

To date, no transcription factor gene is known to be essential for appressorium formation in *M. oryzae*. The *STE12* homolog, *MST12*, is required for appressorial penetration and plant infection (Park et al., 2002). However, the *mst12* mutant still forms melanized appressoria. *MST12* appears to be required for cytoskeleton reorganization associated with appressorium maturation and penetration peg formation. Appressoria formed by the *mst12* mutant fail to develop penetration pegs and elicit plant defense responses in the underlying plant cells. Both the N-terminal STE domain and two C-terminal tandem zinc finger motifs are well conserved and essential for the *MST12* function (Park et al., 2004). The middle region of *MST12* contains putative PKA and MAPK phosphorylation sites and may function as the modulation domain. In yeast two-hybrid assays, Mst12 interacts weakly with Pmk1. *MST12* may function as one of the downstream transcription factors of *PMK1* for appressorial penetration and infectious growth.

Because *PMK1* is essential for appressorium formation, transcription factor(s) other than *MST12* must be regulated by the *PMK1* pathway for appressorium formation. One of the candidates is *PTH12*, which encodes a homeobox-containing protein. A REMI mutant disrupted in the *PTH12* gene is defective in appressorium formation on conducive surfaces (Sweigard et al., 1998). However, the *pth12* deletion mutant still occasionally forms appressoria and causes blast lesions on rice seedlings. Exogenous cAMP also can induce appressorium formation on hydrophobic or hydrophilic surfaces in the *pth12* mutant (Prof. Y. L. Peng, personal communication). Therefore, *PTH12* likely functions downstream from the cAMP-PKA pathway for surface recognition. However, the interaction between the cAMP signaling and *PMK1* MAPK pathways during appressorium formation is not clear.

It remains possible that *PTH12* and *MST12* are components of transcription factor complexes that are regulated by the *PMK1* pathway (Fig. 1). In *S. cerevisiae*, Ste12 interacts with transcription factors Tec1 and Mcm1 to regulate filamentous growth and mating processes, respectively. The *M. oryzae* genome has the *MCM1* and *TEC1* homologs but their interaction with *MST12* has not been examined.

9 Genes Regulated by the PMK1 Pathway

To identify genes regulated by *PMK1*, RNA samples were isolated from conidia of wild-type Guy11 and the *pmk1* mutant nn78 that were allowed to germinate on wax paper for 18 h. A subtractive cDNA library enriched for genes regulated by *PMK1* was constructed with Guy11 as the tester and nn78 as the driver (Xue et al., 2002). Among the genes identified by sequence analysis of this library are homologs of *M. oryzae* appressorium ESTs, enzymes involved in sugar transport or metabolism, enzymes related to lipid metabolism, secreted proteins, regulatory proteins, enzymes for secondary metabolism, and hypothetical proteins. Two of them, *GAS1* and *GAS2*, have been functionally characterized (Xue et al., 2002). The *gas1*, *gas2*, and *gas1 gas2* double mutants have no defect in growth and appressoria formation, but are reduced in appressorial penetration and virulence. Both *GAS1* and *GAS2* are specifically expressed during appressorium formation but have no specific subcellular localization patterns (Xue et al., 2002).

Comparative analyses of ESTs sequenced from cDNA libraries of wild-type appressoria and *pmk1* germlings (Ebbole et al., 2004) also have been used to identify genes with altered expression levels in the *pmk1* mutant (Ebbole et al., 2004; Soanes and Talbot, 2005). However, none of these putative *PMK1*-regulated genes identified by computational analyses has been examined experimentally to determine their expression patterns or functions. Recently, microarray analysis with the *M. oryzae* whole genome array (Dean et al., 2005) has been used to identify genes regulated by *PMK1* with RNA samples isolated from appressoria formed by the wild type and germlings of the *pmk1* mutant on a plastic surface (Ding and Xu, unpublished). In comparison with the wild type, 1803 and 1048 genes were up- and down-regulated, respectively, over two-fold in the *pmk1* mutant. The down-regulated genes include *PTH11* and seven genes with a putative MAP kinase docking site.

Overall, the *PMK1* pathway regulates infection-related processes that do not exist in *S. cerevisiae*. The *PMK1* homologs also have been shown to be important for pathogenesis in over a dozen other plant pathogenic fungi, including *Cochliobolus heterostrophus, Colletotrichum lagenarium*, and *Fusarium graminearum* (Xu, 2000; Zhao et al., 2007). In addition to their role in appressorium-forming fungi, homologs of *PMK1* also play essential roles in plant infection in root or vascular pathogens *F. oxysporum* and *Verticillium dahliae*, necrotrophs *Botrytis cinerea* and *Alternaria brassicicola*, and the biotrophic pathogen *Claviceps purpurea*, indicating that the *PMK1* pathway has evolved to regulate plant infection in phytopathogenic fungi. While the Mst11-Mst7-Pmk1 MAP kinase cascade is well conserved in

ascomycetous plant pathogenic fungi, this pathway regulates various infection or developmental processes in different fungi. Therefore, the upstream signal inputs and downstream target of this well conserved MAP kinase cascade may be different between *M. oryzae* and *S. cerevisiae* or other fungal pathogens. In *M. oryzae*, *PMK1* is required for appressorium formation, penetration, and infectious growth. It may respond to different signals at different stages of plant infection to regulate specific sets of downstream target genes. Therefore, the identification and characterization of key receptors and transcription factors of the *PMK1* MAPK pathway will be important objectives for future research. Additional components of the *PMK1* pathway may exist and be responsible for the activation of Mst11, signaling specificity, and stabilization of the Mst7-Pmk1 interaction during appressorium formation.

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Surface Sensing and Signaling During Initiation of Rice-Blast Disease

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Abstract Conidial germ tubes of the rice-blast fungus *Magnaporthe grisea* must differentiate into infection structures, called appressoria, in order to penetrate the host. Apart from hydrophobicity, the other host-surface characteristics responsible for appressorium initiation are poorly understood. In this review, we highlight the recent developments in surface sensing and cell signaling important for *Magnaporthe* pathogenesis. Surface rigidity-based mechanotransduction involved in appressorium initiation has been identified recently, together with a heterotrimeric G-protein cascade as a downstream effector module. Critical hardness necessary for initiation of appressoria in *Magnaporthe* has been estimated. Chemical genetic studies and global transcriptome analyses related to surface hardness have indicated that thigmo-morphogenesis is initiated within two hours after conidial germination and is essential for the accumulation of second messengers such as cyclic AMP. Preliminary studies also suggest a possible role for stretch-activated ion channels and a non-canonical G-protein-coupled receptor in hardness sensing and host penetration. These findings are discussed along with a possible function for G proteins in elaborating the extracellular matrix during pathogenic development in *Magnaporthe*.

Keywords *Magnaporthe* · Fungal pathogenesis · RGS · G proteins · Mechanotransduction

1 Preface to Cell Signaling and Heterotrimeric G Proteins

"Signal transduction" describes how individual cells perceive, process, and ultimately transmit information derived from external "signals", such as hormones, drugs, or even light (Rodbell 1995). All eukaryotic cells have the capacity to respond to various chemical and sensory cues from the environment. Heterotrimeric

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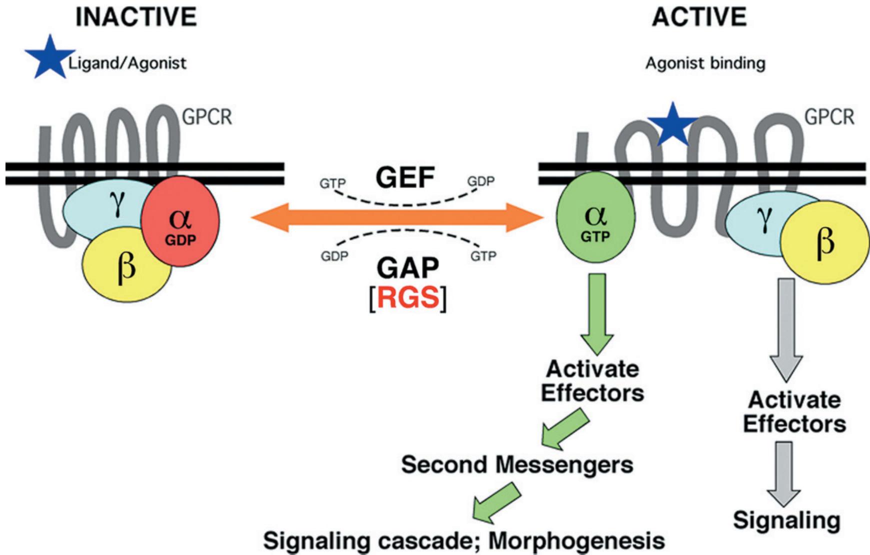

Fig. 1 Schematic representation of canonical G protein signaling. Gα-GDP and Gβγ heterodimer form the inactive G protein complex attached to the cytoplasmic loops of seven-transmembrane receptors (GPCR). Binding of a ligand or agonist to G protein coupled receptor stimulates signal onset by acting as guanine nucleotide exchange factor (GEFs) for the Gα subunit, facilitating GDP-GTP exchange and resulting in the release of the Gβγ dimer. The GTP-bound Gα and the liberated Gβγ moieties are then able to modulate the activity of downstream effectors. RGS proteins (Regulator of G-protein signaling) trigger signal termination by acting as GTPase-accelerating proteins (GAPs) and dramatically enhancing the intrinsic rate of GTP hydrolysis in the Gα subunit

G-protein mediated signaling (Fig. 1) is one of the most important mechanisms by which extracellular stimuli are perceived and converted into intracellular signals. The evolutionary conservation of G-protein signaling in eukaryotes has been a remarkable finding from research done in the last few decades (Dowell and Brown 2002). Such G-protein signaling is often used to regulate transcription activators or repressors to control cell function and development (Hoffman 2005).

Heterotrimeric G proteins are composed of non-identical alpha, beta and gamma subunits (Fig. 1), which mediate signaling from a superfamily of heptahelical receptors (so called G-protein-Coupled Receptors or GPCRs) to a smaller number of downstream effectors that include adenylyl cyclase, phospholipase C and various ion channels (Malbon 2005). These molecules derive their names from the typical three-subunit composition and the capacity of a Ras-like domain in the G-alpha subunit to bind the guanine nucleotides GDP or GTP (Hamm 1998). Based on the sequence similarity and biological function, the G-alpha subunits are classified into four families: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12}$. Functional analyses reveal that $G\alpha_s$ is involved in stimulation of adenylyl cyclase; $G\alpha_i$ inhibits adenylyl cyclase and activates G-protein coupled inwardly rectifying potassium channels (GIRK). $G\alpha_q$ subunit is coupled to the activation of phospholipase C, whereas $G\alpha_{12}$ activates Rho factors (Pierce et al. 2002). As depicted in Fig. 1, heterotrimeric G proteins act as

efficient molecular switches for signal transduction. When the Gα subunit is bound to GDP, docking sites for the downstream effectors are blocked by the Gβ and Gγ subunits. When GDP is exchanged for GTP, three so-called switch regions change their conformation such that Gβ and Gγ subunits dissociate and thus allow downstream effectors to bind the active Gα molecule (Hampoelz and Knoblich 2004). Binding of the cognate ligand to the GPCR promotes rapid conformational changes in the transmembrane helices resulting in the exchange of GDP with GTP at the Gα subunit and dissociation of the heterotrimer (Bourne 1997). Either GTP-bound Gα or the released Gβγ, or both, are then free to activate downstream effectors (Fig. 1; Dohlman 2002). Typically, these effectors produce second messengers or other biochemical changes that stimulate downstream protein kinase cascades. The resultant changes in protein phosphorylation can affect metabolism, ion flux, gene expression, cell morphology, cell movement, cellular differentiation and organismal development (Pierce et al. 2002). Hydrolysis of the GTP by the Gα intrinsic GTPase activity, which can be accelerated by regulator of G-protein signaling (RGS), results in the replacement of GTP with GDP and re-association of the Gα with Gβγ heterodimer, thus leading to the termination of G-protein signaling (Fig. 1). In fungi, G proteins are involved in the regulation of a variety of cellular functions in vegetative growth and/or pathogenic development, such as conidiation, infectious structure differentiation and pathogenicity (Bolker 1998; Lengeler et al. 2000; Yu et al 2006).

2 Signal Perception and Transduction for Appressorium Initiation

In *Magnaporthe grisea*, the asexual spores or conidia do not remain viable for extended periods and must attach quickly to a host leaf to initiate infection (Talbot 1995). Upon sticking to the leaf surface by releasing adhesive mucilage (Hamer et al. 1988) from the tip, the conidia start to germinate. Signaling between pathogenic fungi and plants begins when such attachment occurs on the plant surface (Kolattukudy et al. 1995). The conidial germ tube that emerges is a highly specialized structure (Bourett and Howard 1990), distinct from the vegetative fungal hyphae, that sometimes grows for only a short time before differentiation (Mendgen et al. 1996). The germ tube represents the site and developmental stage for the perception of the host surface (see Fig. 2; Bourett and Howard 1990; Beckerman and Ebbole 1996). If appropriate physical and chemical signals are detected by the germ tube, then a complex morphogenesis program is induced, resulting in appressorium formation (Tucker and Talbot 2001; Ahn et al. 2004). However, if environmental signals are not perceived correctly, the germ tubes will remain undifferentiated and will eventually arrest growth upon nutrient depletion. Germ tube extension and differentiation can occur in response to a number of signals, of which surface hydrophobicity has been implicated in triggering appressorium development (Lee and Dean 1994). Fungal hydrophobins are important for appressorium development, possibly by mediating the interactions of the germ tube with hydrophobic surfaces

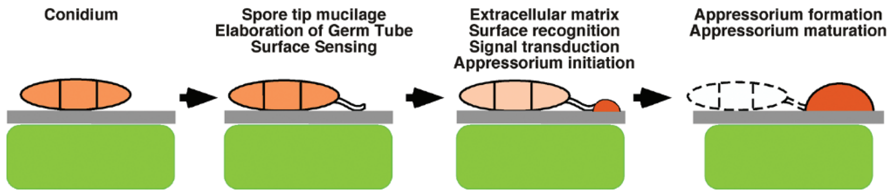

Fig. 2 Surface dependent signaling and *Magnaporthe* development. Schematic diagram describing two important phases in the life cycle of *Magnaporthe*: Vegetative phase (*upper panel*) and the pathogenic phase (*lower panel*). Conidial germination and development on non-inductive surface involves three stages: Dissemination of conidium on non-host surface, elaboration of germ tube, and differentiation into mycelial growth phase. Pathogenic phase (inductive surface) describes the establishment and initiation of the infection process in *Magnaporthe*. Important and key development events associated with each stage of differentiation (upon proper surface recognition) are described in each instance

to initiate the early signaling (Talbot et al. 1993; Beckerman and Ebbole 1996). The first process in signal transduction is the perception of an extracellular signal and its transmission via the plasma membrane, resulting in accumulation of intracellular signaling molecules and cellular response (Tucker and Talbot 2001; Kamakura et al. 2002). A putative membrane-bound sensor or receptor, encoded by the *PTH11* gene, has been identified in *Magnaporthe* and appears to respond to the presence of hydrophobic surfaces, transmitting a cue for appressorium formation (DeZwaan et al. 1999; Tucker and Talbot 2001). Pth11 is a plasma membrane protein with a CFEM domain at the N-terminus and eight transmembrane helices, but is suggested to serve as a GPCR (DeZwaan et al. 1999; Kulkarni et al. 2005). Pth11-deficient strains are non-pathogenic due to a defect in appressorium differentiation (DeZwaan et al. 1999). On inductive surfaces, functional appressoria are formed by $pth11\Delta$ but only at 10–15% of the wild-type frequency, suggesting that Pth11 is not essential for appressorium morphogenesis but is involved in host surface recognition and/or response (DeZwaan et al. 1999). Pth11 likely functions at the cell cortex as an upstream effector or as a cell surface receptor, for appressorium differentiation in response to surface hydrophobicity (DeZwaan et al. 1999).

3 Intracellular Signal Transduction

In contrast to limited knowledge about the perception of external cues, the intracellular signaling cascades involved in appressorium development and function are relatively well understood. It was reported more than a decade ago that a cyclic-AMP dependent signal transduction pathway is required for appressorium

formation in *Magnaporthe* (Lee and Dean 1993). Exogenous cAMP induces appressoria in *Magnaporthe* conidia germinating on non-inductive surfaces (Mitchell and Dean 1995; Choi and Dean 1997). Following this initial finding, additional components involved in cAMP signaling in *Magnaporthe* were identified and characterized in detail. An adenylyl cyclase enzyme, Mac1, essential for appressorium formation and pathogenicity has been well characterized (Choi and Dean 1997). Exogenous cAMP can rescue the defects associated with the loss of *MAC1*. Furthermore, the catalytic subunit of the cAMP-dependent protein kinase A (CpkA) was identified (Mitchell and Dean 1995), and it was found that appressorium development is delayed and inefficient in the $cpkA\Delta$ mutant (Xu et al. 1997; Adachi and Hamer 1998). These results suggest an important role for cAMP accumulation (and signaling) at the initial stages of appressorium initiation in *Magnaporthe*.

In addition to the cAMP signaling pathway, the roles of MAP kinase cascades in appressorium development have also been well studied in *Magnaporthe* (Xu and Hamer 1996). *Magnaporthe* Pmk1 shows extensive similarity to *S. cerevisiae* MAP kinases Fus3 and Kss1, and functionally complements the mating defect of $fus3\Delta kss1\Delta$ double mutant (Xu and Hamer 1996). Loss of *PMK1* leads to a complete cessation of appressorium formation and to non-pathogenicity (Xu and Hamer 1996). The $pmk1\Delta$ mutant remains responsive to cAMP for germ tube hooking and swelling, the early symptom of appressorium differentiation (Xu and Hamer 1996; Tucker and Talbot 2001). Based on these observations, it has been proposed that Pmk1 acts downstream of cAMP signaling for appressorium formation, although a direct interaction between components of these two pathways has yet to be identified (Dean 1997; Xu 2000). *Magnaporthe* Mps1, related to the yeast Slt2 MAP kinase, is essential for host penetration and invasive growth (Xu et al. 1998). This suggests that Mps1 is not important for appressorium development per se, but is critical for its function (Xu et al. 1998).

4 Heterotrimeric G Proteins in *Magnaporthe*

Heterotrimeric G proteins regulate a variety of cellular functions during sexual and pathogenic development in fungi (Bolker 1998). G proteins serve as critical transducers between activated cell surface receptors and intracellular effectors such as cAMP (Gilman 1987; Papasavvas et al. 1992). Three distinct Gα subunits (MagA, MagB, and MagC), two Gβ subunits (Mgb1 and Mgb2) and one Gγ subunit are present in *Magnaporthe* (*Magnaporthe* genome database, Broad Institute, USA; Dean et al. 2005). While MagA and MagB represent $G\alpha_s$ and $G\alpha_i$, respectively, MagC belongs to the fungal-specific $G\alpha_{II}$ class of G-alpha subunits (Bolker 1998; Liu et al. 2007). Neither $magA\Delta$ nor $magC\Delta$ mutants show any defects in vegetative growth or appressorium formation (Liu and Dean 1997; Liu et al. 2007). However, disruption of *MAGB* significantly reduces vegetative growth, conidiation and appressorium formation (Liu and Dean 1997). Moreover, a dominant-active mutation of MagB ($magB^{G42R}$) affects growth and appressorium formation in rice-blast (Fang and Dean 2000). The roles of Mgb1, the Gβ subunit, during growth

and infection-related morphogenesis have also been examined in *Magnaporthe*. The *mgb1*Δ mutant lacks appressoria, whereas elevated Mgb1 protein levels promote appressoria even on non-inductive surfaces (Nishimura et al. 2003). These results underpin the importance of G proteins in several signal transduction pathways such as vegetative growth, conidiation, appressorium formation, pathogenicity and mating in *Magnaporthe* (Liu and Dean 1997; Fang and Dean 2000; Nishimura et al. 2003; Liu et al. 2007).

5 Surface Hardness Stimulus is an Essential Trigger for Appressorium Differentiation in *Magnaporthe*

Magnaporthe conidia represent the causal agents of the devastating blast disease in several monocot species including rice (Ou 1985). When conidia germinate on non-inductive surfaces, the emerging germ tubes do not differentiate into appressorium but grow vegetatively to form mycelia (Fig. 2). However, when conidial germination occurs on leaves or artificial inductive surfaces, the tips of the germ tubes attach to the surface and undergo swelling and hooking to initiate appressoria formation (Bourett and Howard 1990; Mendgen et al. 1996; see Fig. 2). Environmental and physicochemical surface signals that are searched and probed for by the germ tubes, act as critical triggers for appressorium differentiation. For instance, hydrophobic but not hydrophilic membranes are capable of inducing appressoria (Lee and Dean 1994), and hence surface hydrophobicity has been implicated as an important cue for appressorium development. In *Magnaporthe*, response to the hydrophobic surface requires the Mpg1 hydrophobin, which is predicted to interact with hydrophobic surfaces and thus acts as a developmental sensor involved in surface recognition and appressorium initiation (Talbot et al. 1993; 1996). Until recently, it wasn't clear whether hydrophobicity is the only signal perceived for appressorium differentiation or whether other surface characteristics are important as well. Recent analysis of the Regulator of G-protein signaling in *Magnaporthe* (Rgs1; Liu et al. 2007) helped to dissect the key aspects of surface signaling and to uncouple surface dependency from pathogenic differentiation. The signaling for appressorium development was found to be constitutively active in cells lacking Rgs1 (Liu et al. 2007). It has been suggested that surface hydrophobicity alone is sufficient to induce appressorium formation in *Magnaporthe* (Lee and Dean 1994). However, assays performed on artificial surfaces with different physicochemical properties revealed that hydrophobicity alone is not sufficient to induce appressoria, but additional signals, particularly surface hardness (rigidity of about 150 kilopascal), are indeed necessary for efficient appressorial differentiation in *Magnaporthe* (Liu et al. 2007). Rather strikingly, the ability to induce appressorium development was directly related to the surface hardness. Thus, the identification of Rgs1 has helped uncouple the hardness sensing from other inductive stimuli required for appressorium development in *Magnaporthe* (Liu et al. 2007).

6 Thigmotropic Cue and Second Messenger Accumulation

Cyclic AMP (cAMP) has been implicated as a critical mediator of cell signaling during appressorium development in *Magnaporthe* (Mitchell and Dean 1995; Choi and Dean 1997). Loss of adenylyl cyclase Mac1 or of the cAMP-dependent Protein kinase A activity, leads to a failure in appressorium formation (Mitchell and Dean 1995; Choi and Dean 1997). A MAP kinase cascade centered on Pmk1, with possible interplay with cAMP signaling, has also been identified as an essential signaling pathway necessary for pathogenic growth and differentiation (Xu and Hamer 1996; Xu 2000; Bruno et al. 2004).

Magnaporthe conidia begin to germinate immediately upon hydration and short germ tubes are visible within 1–3 h. Swelling and hooking of the tip of the germ tube, which indicates the initiation of appressorium differentiation is usually seen at the 4–6 h stage post inoculation. There seems to be a critical window that perceives and responds to cAMP threshold/signaling in the developmental pathway mentioned above (Beckerman and Ebbole 1996). Interestingly, exogenous cAMP is sufficient to trigger appressorium development only on non-inductive hard surfaces but not on soft surfaces (Liu et al. 2007). This suggests a likely functional relationship between surface perception and the G-protein signaling in *Magnaporthe*. Furthermore, it implies that the accumulation of cAMP is indeed coupled with proper perception and integration of the initial thigmotropic cue during the initiation of appressorium formation (Liu et al. 2007).

7 Summary and Future Prospects

Magnaporthe conidia sense physical and chemical signals from the plant surface to trigger germination and pathogenic differentiation. The G-protein signaling plays an essential role in transducing such surface cues to the downstream effectors for cellular morphogenesis and development. Surface rigidity is a critical trigger for appressorium development. Neither the wild type nor mutants with elevated cAMP signaling (such as *rgs1*Δ) are capable of forming appressoria on soft surfaces regardless of the hydrophobicity, thus suggesting that surface hydrophobicity alone is not sufficient to initiate appressoria. However, contact with a hard surface was found to be necessary for *Magnaporthe* to form appressoria (Liu et al. 2007). The hardness signal was perceived within 2 h upon germination of conidia. Chemical genetic analysis revealed that stretch activated ion channels might be involved in such mechanotransduction during *Magnaporthe* pathogenesis (Liu and Naqvi, unpublished data). It remains to be seen whether the mechanosensitive channels (MscS, small current or MscL, large current) play any role in early thigmotropic signaling during blast disease initiation. It would be interesting to test whether surface rigidity directly activates the G proteins (or the relevant GPCR) in *Magnaporthe*, similar to the stimulatory effect of fluid shear stress on a GPCR in endothelial cells (Chachisvilis et al. 2006).

It is intriguing that constitutive activation of cAMP signaling leads to a severe reduction in *Magnaporthe* pathogenesis (Nishimura et al. 2003; Liu et al. 2007). This is likely due to a direct effect on host penetration efficiency in mutants accumulating higher cAMP levels (Ramanujam and Naqvi, unpublished data). This raises an exciting possibility that enzymes that regulate cAMP hydrolysis, namely the phosphodiesterases, are under negative feedback regulation of the G proteins. Future experiments addressed at identifying the downstream effectors of the G-alpha subunits, particularly MagA and MagB, should provide valuable information on the regulation of such signaling, and would help uncover key regulators and targets of G proteins during the host penetration and invasion stages of rice-blast infections.

Lastly, conidia elaborate spore tip mucilage for attachment, whereas adhesion of germ tubes to the leaf surface requires the secretion of an exracellular matrix, which is poorly understood in *Magnaporthe* (Ahn et al. 2004). It remains to be seen whether these two processes (mucilage and the extracellular matrix) are regulated by feedback mechanisms through the G protein-dependent cAMP signaling. Equally important would be to elucidate the influence (if any) of surface attachment on the induction of thigmotropism and subsequent cAMP signaling in the initiation of appressoria and host colonization in *Magnaporthe*.

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Studies on Autophagy Machinery in *Magnaporthe oryzae*

Fu-Cheng Lin, Xiao-Hong Liu, Jian-Ping Lu and Tong-Bao Liu

Abstract The pathogenic fungus, *Magnaporthe oryzae*, has emerged as a model system for dissecting fungal-plant interactions. Considerable progress has been made in identifying genes necessary for the regulation of pre- and post-penetration events in the development and pathogenicity of the fungus, such as the appressorium (a dome-shaped infection structure) mediated penetration and invasive growth of the pathogen. However, the detailed molecular mechanisms of these developmental stages, especially appressorium morphogenesis, are still relatively poorly understood. Currently, we isolated the *MNH6* gene for a nonhistone chromosomal protein and 24 autophagic genes from the genome of this fungus. These genes are highly conserved among other eukaryotes, including humans and plants, and they control the capability of surviving starvation, conidiation, conidial germination, lipid turnover, and appressorium turgor generation. Clarification of the functions of these autophagy genes and their interaction networks will allow us to understand the role of autophagic genes in fungal pathogenesis.

Keywords *Magnaporthe oryzae* · Appressorium · Autophagy

1 Introduction

As one of the most widespread and devastating plant diseases in the world, rice blast causes significant crop losses, representing a loss of 157 million tons of rice per annum in the staple food crop of one half of the world's population and leading to multiple social crises. A model fungus, *Magnaporthe oryzae* causing rice blast disease, has been well studied worldwide for many years (Dean et al., 2005; Scaffidi, 2002; Tucker and Talbot, 2001). The pathogenic fungus employs a specialized cellular structure, the appressorium (de Jong et al., 1997; Dean, 2005; 1997; Howard, 1991; Scaffidi, 2002; Thomas, 2001), in order to penetrate different host tissues

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such as leaves, stems. Considerable progress has been made in identifying gene functions necessary for the regulation of appressorium-mediated penetration events in the development and pathogenicity of *M. oryzae*, and its genomic sequence has been obtained (Balhadère, 1999; Dean, 2005; Dixon, 1999; Hamer et al., 1988; Ou, 1985). There have been two signaling pathways, the cAMP signaling and PMK1 MAPK signaling pathways, characterized in *M. oryzae* relative to appressorium formation (de Jong et al., 1997; Dean, 1997; Lee and Dean, 1993; Talbot, 2003; Xu and Hamer, 1996; Xu et al., 1997). However, molecular mechanisms in appressorium development or pathogenicity have not been fully characterized for the rice blast fungus (Talbot, 2003). Here, we report our recent research on molecular biology of appressorium morphogenesis in *M. oryzae*, mainly related to the autophagy pathway.

2 Autophagic Genes Express During Appressorium Formation

We have developed a novel method for purification of total RNA from fungal appressoria of *M. oryzae*. The total RNA of appressoria was isolated directly from the appressoria of this fungus inoculated on a hydrophobic surface of projection transparency film (terylene resin membrane, Gaoke, China) with the Trizol reagent. Using this technique, we successfully obtained the total RNAs from appressoria incubated for 2h, 4h, 8h, 12h, 18h, 24h, and 30h after conidial germination and built separate appressorium cDNA libraries. Total RNA from a conidia/aerial mycelia/substrate mycelia mixture and mature appressoria, incubated for 23.5–24.5h, was isolated separately and a subtractive appressorial cDNA library, subtracted by the conidial and mycelial mixture, was constructed by SSH. Among 142 ESTs selected, 70 ESTs were described previously and another 72 ESTs were reported for the first time in *M. oryzae*. Eighty-nine percent (when compared with protein databases) of the 142 ESTs had significant matches ($p < 0.001$) to known (or predicted) genes present in GenBank at the time of submission. All 142 identified genes were organized into functional classifications. Forty-nine percent of ESTs were classified as having an unknown function. Twelve ESTs (8%) were assigned as fat metabolism genes such as cytochrome P450, keto acyl reductase, acyl-CoA dehydrogenase, autophagy pathway genes, HMGB and C-14 sterol reductase. And ten ESTs (7%) were assigned to protein modification such as serine-theronine kinase, oligosaccharyltransferase alpha subunit, and cysteine proteinase. The remaining genes were grouped into nucleic acid metabolism (7%), sugar metabolism (6%), antibiotic metabolism (4%), protein synthesis (3%), cell wall (1%), and other functions (15%). We also used RT-PCR analysis to confirm differential gene expression in appressoria and conidia/aerial mycelia/substrate mycelia as indicted by the SSH subtraction strategy. These results indicate that our SSH results are successful in revealing differential gene expression in the appressorial stage of *M. oryzae*. Functional analyses of 35 novel genes related to this stage have been performed (Lu et al., 2005a,b).

3 Autophagy Genes

Autophagy is a ubiquitous and evolutionarily conserved process that degrades and recycles long-lived proteins and organelles, occurring in all eukaryotic cells. It is an intracellular, bulk degradation process in which cytosol and organelles are sequestered within double-membrane vesicles termed autophagosomes that deliver the contents to the lysosome/vacuole for degradation and recycling of the macromolecules (Klionsky, 2005; Levine and Klionsky, 2004). For many years, autophagy has been presumed to be involved in cellular architectural changes that occur during differentiation and development, presumably via its role in organelle and protein turnover (Kikuma et al., 2006; Veneault-Fourrey et al., 2006). We found that some autophagy genes were up-regulated during appressorium maturation, and identified 24 putative *M. oryzae* autophagy genes that are homologous to the yeast genes (Table 1).

An EST corresponding to *MgATG1* (Genbank Accession No. CK828251), encoding a serine/threonine kinase required for autophagy, was found in screening genes highly expressed during the appressorium stage from the subtractive suppressive cDNA library of 24h mature appressoria. To identify the potential role of *MgATG1* during appressorium formation, maturation and penetration, we isolated the full-length *MgATG1* gene from genomic DNA of *M. oryzae* strain Guy11 and the appressorium stage cDNA library by long distance PCR. The 2949bp cDNA fragment containing the full coding DNA sequence (CDS) was cloned into pUCm-T (Sangon, Shanghai, China) and sequenced (Genbank Accession No. DQ224381).

MgATG1 in *M. oryzae*, containing a S_TKc domain of 256 residues, is a 982-amino acid serine/threonine protein kinase with a calculated molecular mass of 108 kDa. The alignment between *MgATG1* of the rice blast fungus and *ATG1* from other organisms shows very high homologies. Significantly, ATG1 in *M. oryzae*

Table 1 Orthologs of yeast ATG genes in *M. Oryzae*

ATG	*M. oryzae*	ATG	*M. oryzae*
ATG1	MGG_06393.5	ATG13	MGG_00454.5
ATG2	MGG_05998.5	ATG15	MGG_12828.5
ATG3	MGG_02959.5	ATG16	MGG_05255.5
ATG4	MGG_03580.5	ATG17	MGG_07667.5
ATG5	MGG_09262.5	ATG18	MGG_03139.5
ATG6	MGG_03694.5	ATG20	MGG_12832.5
ATG7	MGG_07297.5	ATG21	MGG_03139.5
ATG8	MGG_01062.5	ATG22	MGG_09904.5
ATG9	MGG_09559.5	ATG23	MGG_10579.5
ATG11	MGG_04486.5	ATG24	MGG_03638.5
ATG12	MGG_00598.5	ATG26	MGG_03459.5
VPS15	MGG_06100.5	VPS34	MGG_03069.5

Note: Using the published Atg protein sequences in yeasts, we have screened protein and DNA databases to identify putative functional homologs (orthologs) from the available *M. oryzae* genome sequence.

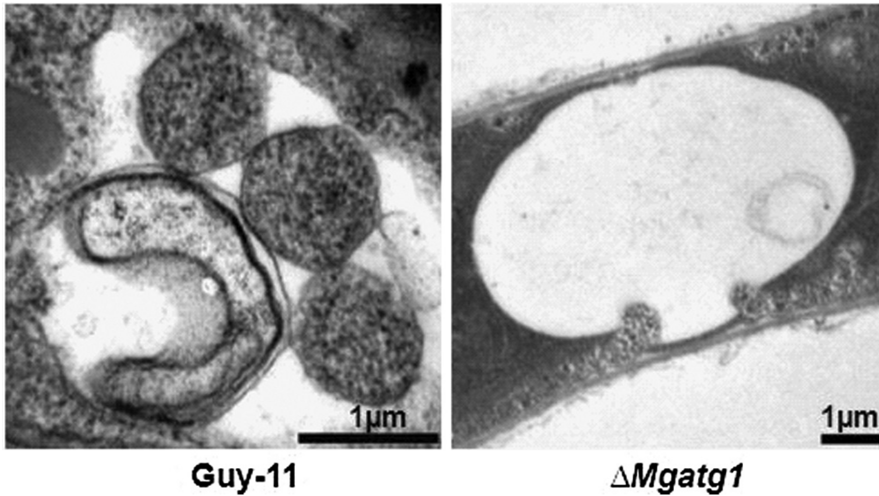

Fig. 1 Autophagic bodies in a vacuole. Numerous autophagic bodies accumulated in the vacuoles of the wild type-Guy-11, but few such bodies were observed in vacuoles of atg null mutant-Δmgatg1

is 60% identical to *MgATG1* (*CLK1*) in *Colletotrichum lindemuthianum*, 56% to *ATG1* (PDD7p) in *Hansenula polymorpha*, 51% to ATG1 (APG1/AUT3) in *Saccharomyces cerevisiae* (Matsuura et al., 1997), and 36% to *ATG1* (*ULK1*) in *Mus musculus*, *ATG1* (*ULK1*) *Homo sapiens* and *ATG1* (*UNC-51*) *Caenorhabditis elegans* (Ogura et al., 1994). The Δ*Mgatg1* mutant of *M. oryzae* Guy11, in which the *MgATG1* gene was deleted by targeted gene replacement, shows a defect in autophagy (Fig. 1) and also exhibits defects in morphogenesis including reduction

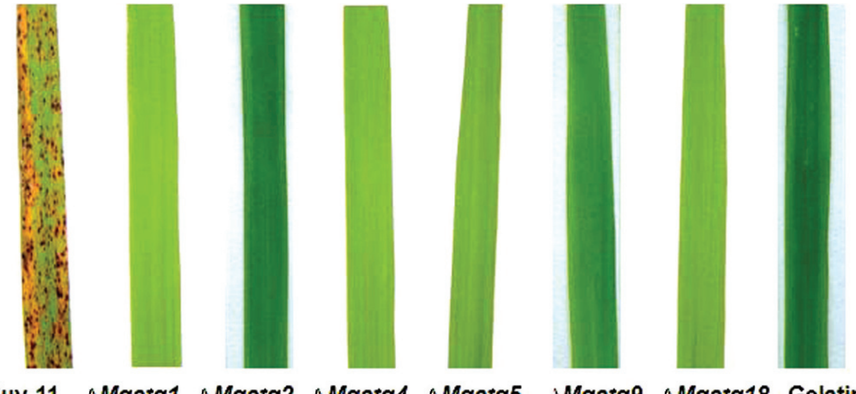

Fig. 2 atg mutants had lost their abilities for penetration of the host plants. Shown are wild type Guy-11; atg mutants Δmgatg1, Δmgatg2, Δmgatg4, Δmgatg5, Δmgatg9 and Δmgatg18; and a Gelatin control

in conidiation, delay in conidial germination, and reduction in appressorial turgor pressure. As a result, the Δ*Mgatg1* mutant had lost its pathogenicity to two tested host plants, rice CO-39 and barley ZJ-8. Wild type phenotypes were recovered by re-introduction of an intact copy of *MgATG1* to the mutants. Taken together, the *MgATG1* gene is essential for autophagy and is also required for development and pathogenicity of *M. oryzae* (Liu et al., 2007a,b). Other genes, such as *MgATG2*, *MgATG4*, *MgATG5*, *MgATG9* and *MgATG18* in the autophagy pathway, were also found to be related to appressorium development and pathogenicity of this fungus (Fig. 2).

4 Nonhistone Chromosomal Protein *MNH6* Gene May Be Related to Autophagy Pathway

By analyzing genes in a subtractive suppressive cDNA library of mature appresoria in *M. oryzae*, we have cloned a gene, *MNH6* (Lu et al., 2007), homologous to *NHP6A/B* of *S. cerevisiae* (Biswas et al., 2006), using a PCR-based strategy and have shown by targeted deletion that this gene is important for growth, development, and pathogenicity. According to the protein sequence, MNH6, *Magnaporthe* nonhistone protein 6, is related to the baker's yeast HMG1-like chromatin-associated proteins NHP6A and NHP6B. These proteins belong to the HMGB family of the HMG superfamily, whose main function in other eukaryotes covers transcription regulation, DNA repair, recombination, differentiation, and extracellular signaling (Baxevanis and Landsman, 1995; Bianchi and Agresti, 2005; Biswas et al., 2006; Costigan, 1994; Dalrymple and Peters, 1992; Fragiadakis, 2004; Kim et al., 2005; Kolodrubetz and Burgum, 1990; Kruppa and Kolodrubetz, 2001; Lu et al., 2007; Marchler-Bauer and Bryant, 2004; Moreira and Holmberg, 2000; Muller et al., 2001; Scaffidi, 2002; Thomas, 2001).

Using a gene-deletion mutation in the *MNH6* locus (Kruppa and Kolodrubetz, 2001; Lu et al., 2007), we have uncovered an important and essential role for this nonhistone chromosomal protein in the pathogenesis cycle of the rice blast fungus. During the vegetative growth phase, Δ*mnh6* mutants show aberrant mycelial growth and conidiation. Δ*mnh6* mutant mycelia grow at 80% of the wild type level and differentiate conidia at 50% compared to the wild type strain. The shape of mycelia and conidia in Δ*mnh6* mutants has changed. Hyphae are compact and dark gray to spare off-white, and conidia vary from pyriform to slender compared with wild type strain Guy11. The ability to form appressoria from germinated conidia in Δ*mnh6* mutants is reduced to 70% and the appressoria that form are also smaller than Guy11 appressoria. As Δ*nhp6* mutant cells of yeast display defects in actin polarity and the cytoskeleton, the morphological defects in mutants may be caused by the those defects in actin polarity and cytoskeleton. It is possible that the defects in growth morphology of Δ*mnh6* mutants also result from defects in actin polarity and cytoskeleton. However, the frequency of conidial germination in Δ*mnh6* mutants maintains the high level of the wild-type strain, and Δ*mnh6*

mutants form viable ascospores when incubated with the opposite mating-type strain 2539. The fact that the *MNH6*-complemented strains recovered wild type characteristics for vegetative growth, conidiation and appressorium formation proved that the phenotypic defects observed in the $\Delta mnh6$ mutants resulted directly from deletion of this gene. We conclude that *MNH6* plays important pleiotropic roles in the growth and morphogenesis of mycelia, conidia and appressoria. These pleiotropic effects on fungal morphogenesis exhibited by $\Delta mnh6$ mutants strongly support that *MNH6* is required for effective pathogenicity and completion of the rice blast disease cycle. Further analysis of *MNH6* and its relationship with other known pathogenesis genes through affecting transcriptional regulation in this fungus, and regulation of downstream biological cascade events, including the autophagy pathway, cAMP-dependent and/or MAP kinase pathways (Xu and Hamer, 1996; Xu et al., 1997), may yield insights into morphogenesis and pathogenicity in the rice blast fungus. Microarray analyses may provide novel clues for investigation of the networks of the downstream pathway related to autophagy in this fungus.

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Secretion Property and Gene Expression Pattern of a Putative Feruloyl Esterase in *Magnaporthe grisea*

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Abstract Plant cell walls are a pivotal battleground between microbial pathogens and their plant hosts. Microbial pathogens secrete an array of cell-wall-degrading enzymes to depolymerize the noncellulosic polysaccharides of primary cell walls, which are essential for the vitality of pathogens, either saprophytically, pathogenetically or both. The predicted protein encoded by *MGG_01403.5* from *Magnaporthe grisea* has a high degree of sequence identity with the ferulic acid esterase A (so to be designated as MgFaeA) from *Penicillium funiculosum*. Tested by the SignalP, TMHMM and Protcomp programs, the 284-amino acid hypothetical protein contained a cleavable signal peptide at the N terminus, lacked transmembrane structure, and appeared located in the extracellular matrix. In the work presented here, MgFaeA, with a His6 tag at its C-terminus, was introduced into *M. grisea* for overexpression. The MgFaeA-His6 fusion protein was purified from the culture filtrate, which confirmed the secretion property of MgFaeA in the fungus. To further investigate the role of MgFaeA on rice infection, RT-PCR was used to analyze the gene expression during different stages of infection. The results indicated that MgFaeA transcripts were detectable as early as 72 hpi, and reached to peak at 7 dpi. It suggested that the MgFaeA gene might be involved in the fungal pathogenesis.

Keywords Ferulic acid esterase · *Magnaporthe grisea* · Secreted protein

1 Introduction

Any micoorganism that intends to invade living plant tissue must contend with the cell wall, and all major groups of plant pathogens are known to produce extracellular enzymes that can depolymerize polysaccharides in primary cell walls of plants. These cell wall-degrading enzymes are usually postulated to play specific roles in penetration and ramification once a pathogen is inside the plant, and in

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the release of nutrients to support fungal growth (Ten Have et al. 1998; Tonukari et al. 2000; Walton, 1994). Furthermore, some microbial wall depolymerases also elicit plant defense responses. For instance, endopolygalacturonase induced the biosynthesis of phytoalexins (Cervone et al. 1987). Xylanases from several fungi can elicit electrolyte leakage, necrosis, ethylene biosynthesis, and synthesis of pathogenesis-related proteins (Bailey et al. 1990; Dean & Anderson, 1991).

However, many individual structural genes encoding important cell wall-degrading enzymes do not make a major contribution to pathogen virulence due to their redundancy. For example, the pea pathogen *Nectria haematococca* has four functional pectate lyase genes, the maize pathogen *Cochliobolus carbonum* and the rice pathogen *M. grisea* each have at least four xylanase genes (Apel-Birkhold & Walton, 1996; Guo et al. 1996; Wu et al. 1997; 2006). It is likely that pathogens make cell wall depolymerases that are specific for particular uncommon linkages in the native wall, and it is possible that the disruption of these linkages is critica1 for a successful pathogen invasion.

In plant cell walls, polysaccharide and lignin polymers are commonly linked by hydroxy derivatives of cinnamic acid, such as ferulic and p-coumaric acids (Reid, 2000). Ferulic acid residues of gramineous plants are linked mainly to xylan and pectin and may cross-link cell wall polymers, which would increase cell wall rigidity and hence reduce biodegradability by plant-invading microorganisms (Ishii, 1997). Feruloyl esterases are enzymes that release ferulic acid, one of the aromatic residues, from plant cell wall polymers (de Vries et al. 1997). Recently, it was demonstrated that ferulic acid esterase contributes substantially to the lignocellulose-degrading potential of *A. pullulans* (Rumbold et al. 2002).

M. grisea, the causal agent of the devastating rice blast disease (Talbot, 2003), produces extracellular hydroxycinnamic ester-hydrolyzing enzymes, including feruloyl esterases, with activity against cross-linking between polysaccharides and lignin in the plant cell wall. As a first step towards analyzing the role of these enzymes in the fungal pathogenesis, the homologous sequence analysis, overexpression, and expression patterns during fungal infection of a putative ferulic acid esterase encoding gene was investigated.

2 Materials and Methods

2.1 Over-Expression of MgFaeA

The *M. grisea* field strain FJ95054B was used as host for the over-expression of *MgFae*A. The $^{\triangle}$*faeA* knock-out mutants were generated and stored in our laboratory. The *Saccharomyces cerevisiae* strain W303 was used as the host strain for yeast recombination cloning. *Escherichia coli* strain DH10B was used as a host for plasmid amplification.

An oligonucleotide encoding the peptide sequence tag RGSHHHHHH ($RGSH_6$) was added to the pTE11 vector to construct pDL1 (Lu et al. 2004) (Figures not shown). Coding regions cloned in-frame with the start codon and $RGSH_6$ tag will be

expressed under the control of the *M. grisea* Ribosomal Protein 27 (RP27) promoter and the expressed protein can be purified using Ni-NTA affinity chromatography.

The full encoding region of *MgFaeA* was amplified by PCR from the 70-15 genomic DNA. PCR products were cloned into the expression vector by homologous recombination in yeast cells (Lu et al. 2004). The constructed plasmid DNA was recovered with a miniprep procedure. Plasmid DNA was used to transform protoplasts of *M. grisea*.

2.2 Expression and Detection of the Recombinant MgFaeA

M. grisea transformants were selected for resistance to hygromycin B, and further purified by spreading conidia to obtain pure cultures. The resulting strains were grown in a complete medium (CM) for several days to obtain mycelia, and then transferred to a fresh medium for an additional 6–24 h. Culture filtrates were harvested to obtain extracellular proteins. For protein purification, Ni-NTA affinity purification was used according to the manufacturer's instrutions (Qiagen, Inc.). SDS-PAGE was conducted using standard protocols (QIAexpress Detection and Assay Handbook, Qiagen, Inc.).

2.3 Gene Expression Studies

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis was performed on RNA isolated from (1) mycelium collected from CM, (2) conidia harvested from rice-polish agar medium, (3) cells grown on a cellophane film for 4 h to induce spore germination, and (4) the infected seedlings 24, 48, 72, 96, 168 h postinoculation (hpi). RNA was prepared using the TRIZOL reagent. For reverse transcription reactions, 5 μg of total RNA were used in a 20 μL reaction using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). One microliter of the RT mix was used for semi-quantitative RT-PCR in a 20 μL volume.

3 Results

3.1 Sequence Analysis of the Ferulic Acid Esterase Homologs in M. grisea

Based on a homology BLAST search, we retrieved 4 hypothetical proteins with high homology to ferulic acid esterase, which are encoded by *MGG_01403.5*, *MGG_13625.5*, *MGG_10040.5*, and *MGG_07653.5* in the *M.grisea* genome. We have named these *M. grisea* ferulic acid esterases as MgFaeA, MgFaeB, MgFaeC, and MgFaeD respectively. Interestingly, MgFaeA, MgFaeB, and MgFaeC also belong to glycosyl hydrolase family 62, a family of alpha -L-arabinofuranosidase (EC3.2.1.55), which hydrolyses aryl alpha-L-arabinofuranosides and cleaves arabinosyl side chains from arabinoxylan and arabinan. Whether these hypothetical

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XP_363477|M.grisa          MMSQTRILG-----LAAIF------------LFLGLTEAAR-------- 25
CAC85738|P.funiculosun     -MVKSYIIGAFVLELASVVLGQQSLWGQCGGTGWTGPTTCVSGACCQEQN 49
                             * :: *:*     **:.::              : * * ..

XP_363477|M.grisa          --------------------------------------------SAGCGK 31
CAC85738|P.funiculosun     PYYSQCIQGNCSPASSTSSTSSTKTTTTSASSTTTSTSASGTSLSSGCGK 99
                                                                       *:****

XP_363477|M.grisa          TNTIRNQQYTANINGKQRQYIVRLPDQYDNNKAHKLVFTFHALGGNAQQI 81
CAC85738|P.funiculosun     ALSLKSGTYTTTVAGQQRQYTLTLPSNYNPNKAYQLIFGYHWLGCTMGNV 149
                           : :::.  **:.: *:**** : **.:*: ***::*:* :* ***.  ::

XP_363477|M.grisa          AQGQGGTLAWYGLPPLSNNSAIFVSPNGLNAGVANQGGEDITFIDNMVRT 131
CAC85738|P.funiculosun     VSC------SYYCIQPLAGDNAIFVAPQGLNNGVGNTNGDDIIFTDQMLST 194
                           ..*      ::**: **:.:.****:*:*** **.* .*:** * *:*: *

XP_363477|M.grisa          IEADLCVETSQAFATGFSYGGAISFAWACARGKEVRAIAPISSSQLSGCQ 181
CAC85738|P.funiculosun     LENALCIDETQIYSHCWSYCGAMSYALACARPDVFRAVAVNSGANLSGCS 244
                           :*  **:: :* :: *:*****:*:* **** . .**:* :*.::****.

XP_363477|M.grisa          GGNDPVAFIGQHGTSDSVLPTAGGRAMRDRFVANNGCTPIQP-EPQPNGG 230
CAC85738|P.funiculosun     PGTQPVAYYGQHGVSDTVLPFSLGEGIRDTFVKDDHCTPTNPPAPAAGSG 294
                            *.:***: ****.**:*** : *..:** **::: *** :*  * ...*

XP_363477|M.grisa          RHVKVDYQGCREGYPVSWIIHNGDHNPSQSDQGSNQAFAPAYSWNFFNQF 280
CAC85738|P.funiculosun     THIKTEYSGCDSEHPVWWIAFDGPHEPLATDAGASSSWTPGQIWSFFSLF 344
                            *:*.:*.** . :** ** .:* *:*  :* *:..:::*.  *.**. *

XP_363477|M.grisa          QPQ 283
CAC85738|P.funiculosun     H-- 345
                           :
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Fig. 1 Amino acid sequence alignment of the predicted *M. grisea* MgFaeA with the ferulic acid esterase A from Penicillium funiculosum. Sequences were aligned with CLUSTALX

proteins function as ferulic acid esterases, as glycosyl hydrolase family 62, or as both needs further biochemical clarification.

Further sequence alignment showed that MgFaeA had 44% overall identity with the ferulic acid esterase A (CAC85738.1) from *Penicillium funiculosum* (Fig. 1). BLASTP analysis indicated that the predicted protein MgFaeA has a conserved domain of bacterial poly (3-hydroxybutyrate) (PHA) depolymerases at the N-terminus. The mature peptide, containing 284 amino acids, had a predicted signal cleavage site between amino acid 23 and 24 (TEA-AR) at the N-terminus according to the SignalP program, and it lacks a transmembrane helix structure predicted by TMHMM. This suggested that MgFaeA is a secreted protein. The protein is predicted to be have extracellular localization based on analysis by the program Protcomp.

3.2 Secretion Property of MgFaeA

To confirm whether the MgFaeA is a secreted protein as predicted, a DNA fragment covering the MgFaeA encoding region was amplified by PCR from genomic DNA of *M. grisea* strain 70-15. PCR products were cloned into the expression vector pDL1 by homologous recombination in yeast cells, with a peptide sequence RGSHHHHHH (RGSH6) at the C-terminus of the product. The resulting construct, pDLFaeA, was transformed into protoplasts of *M. grisea* strain FJ95054B. The

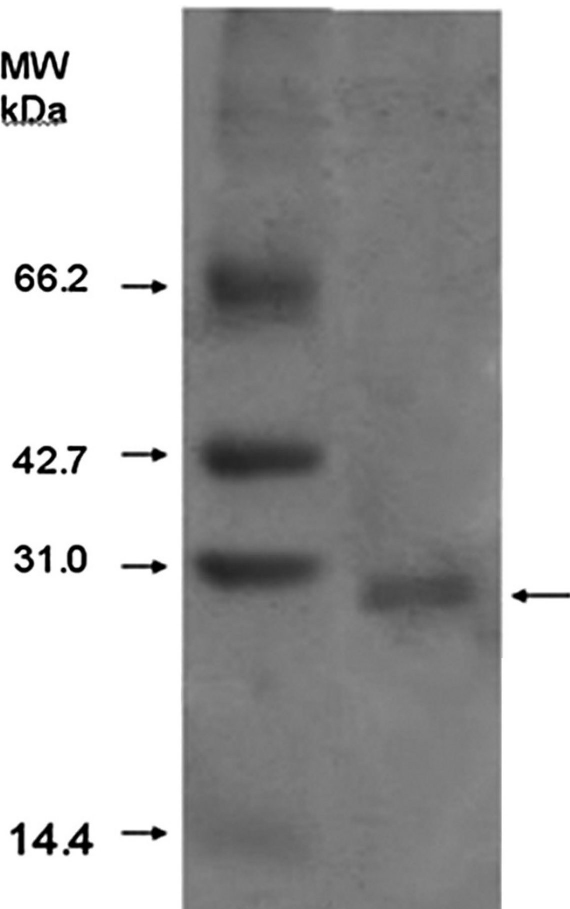


Fig. 2 Purification of recombinant MgFaeA. Purified recombinant MgFAEA was resolved on 5–12% SDS-PAGE. MW was c. 28–29 kDa, indicated by an arrow, similar to the expected MW 28.1kDa

transformants with hygromycin B resistance were selected after PCR confirmation (data no shown). The MgFaeA-His fusion proteins were detected in the culture filtrates of the transformants by SDS-PAGE analysis. The results confirmed secretion of the protein just as predicted by program SignalP. The molecular weight (MW) of the MgFaeA-His fusion protein was 28-30 kDa (Fig. 2), in accordance with the predicted molecular weight (28.1 kDa) of mature MgFaeA.

3.3 Expression of MgFaeA and its Homologs in the Wild Type Strain and Mutants

To further understand the role of MgFaeA in the saprophytic growth and pathogenecity of *M. grisea*, we compared the gene expression patterns in mycelia, conidia, germinated spores and infected rice seedlings by RT-PCR. During vegetative

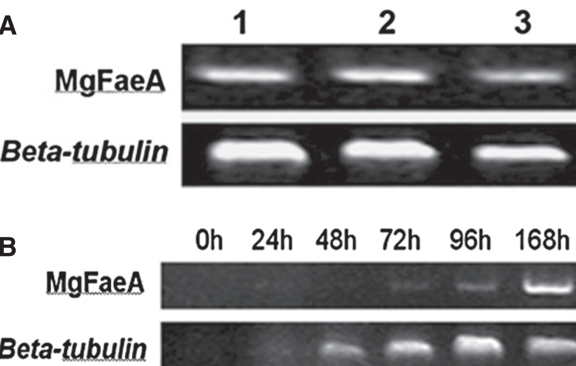
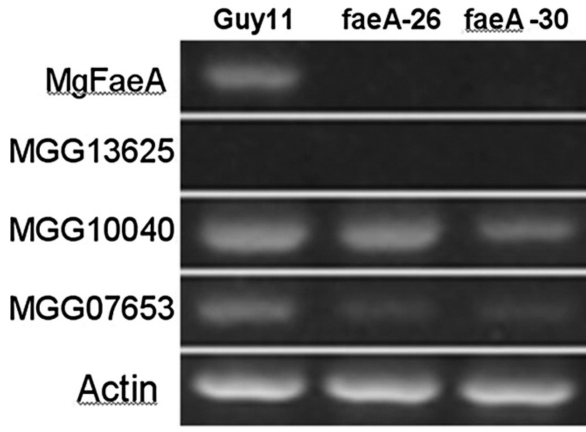


Fig. 3 Expression of MgFaeA at different stages of growth and pathogenecity (**A**) Transcripts of MgFaeA in vegetative growth and asexual development. Lane 1, mycelia growing in CM; lane 2, conidia; lane3, germinated spores (**B**) RT-PCR measurement of MgFaeA expression in infected rice leaves

growth and asexual development, *MgFaeA* transcripts did not show a significant difference (Fig. 3A). However, expression of *MgFaeA* could not be detected in the early stages of rice infection (24 hpi and 48 hpi, Fig. 3B). At this early stage, injury to the plant tissue was barely visible, although conidia from the rice blast fungus had differentiated appressoria and penetrated the rice tissue. At 72 hpi, with the emergence of some small necrotic lesions, the *MgFaeA* transcript was detected, and its abundance reached a maximum at 168 hpi (Fig. 3B), in accordance with the appearance of many expanded lesions.

Gene expression of three MgFaeA homologs in the wild type and knock-out mutants grown in CM was analyzed by RT-PCR (Fig. 4). *MGG_13625* transcripts were undetectable both in the wild type and mutants. The transcripts of *MGG_10040* in the mutants and wild type showed no significant difference, but *MGG_07653* transcripts presented a lower level in the mutants.



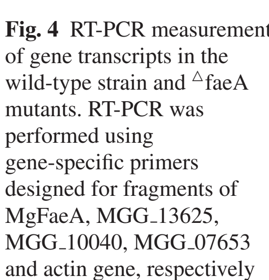


Fig. 4 RT-PCR measurement of gene transcripts in the wild-type strain and △faeA mutants. RT-PCR was performed using gene-specific primers designed for fragments of MgFaeA, MGG_13625, MGG_10040, MGG_07653 and actin gene, respectively

4 Discussion

The recent publication of the *M. grisea* genome sequence has greatly facilitated the elucidation of molecular mechanisms of interaction between a major crop killer and its plant host (Dean et al. 2005). Moreover, a high throughput systematic approach has been applied to detect the secreted proteins from the rice blast fungus, which would help to identify the fungal factors involved in the interaction with the plant host (Chen et al. 2006). Cell wall-degrading enzymes are a large group of proteins secreted by pathogens, including *M. grisea*, which may or may not be involved in pathogenicity. In this paper, *MgFaeA*, encoding a putative ferulic acid esterase was characterized.

Many hydrolases involved in the degradation of plant cell walls are modular in structure. They are comprised of distinct domains, usually including catalytic and binding domains, which are separated by short and often highly glycosylated linker regions (Gilkes et al. 1991). Examples are the acetyl xylan esterase from *Trichoderma reesei*(Margolles-Clark et al. 1996), FaeB from *P. funiculosum* (Kroon et al. 2000) and XYL-6 from *M. grisea* (Wu et al. 2006). Although MgFaeA shows the highest sequence similarity with the fungal cellulose binding domain (fCBD) of *Aspergillus clavatus* protein NRRL1, its N-terminal domain is not similar to a cellulose binding carbohydrate-binding module (CBM). Instead it presents high similarity with bacterial poly(3-hydroxybutyrate) (PHA) depolymerases, which is included in a small family of serine esterases (Kroon et al. 2000).

The major obstacle to identification of the cell wall depolymerases is that most of their expression is under tight regulation, i.e. catabolism product repression and substrate induction. An effective approach to tackle this problem is to harvest the target protein fused with one or more purification tags, under the control of a constitutive promoter in an appropriate expression host. The high levels of protein expression driven by promoter RP27 and the efficient purification of the MgFaeA:His6 tag protein by one-column affinity chromatography make it possible to investigate the enzymatic mode of action and substrate specificity of MgFaeA (Faulds et al. 2005; Rumbold et al. 2003).

Redundancy is emerging as a common theme among the cell-wall degrading enzymes of plant-pathogenic bacteria and fungi (Apel-Birkhold & Walton, 1996; Guo et al. 1996; Wu et al. 1997; 2006), and the ferulic acid esterases of *M. grisea* are not an exception. Our BLAST search of the *M. grisea* genome database showed three hypothetical proteins with high homology to MgFaeA. Structurally, they all contain a conserved domain with significant similarity to bacterial PHA depolymerases, among which MGG_13625 and MGG_10040 include an N-terminal and C-terminal fCBD, respectively. However, RT-PCR analysis of these genes transcripts in the wild type and $\Delta faeA$ mutants revealed that their expression patterns were different. The *MGG_13625* transcripts can not be detected in mycelia of wild type and mutant strains. Our preliminary results showed that the deletion of *MgFaeA* did not affect *MGG_10040* expression, but down-regulates the expression of MGG_07653. These results provide two implications: (i) The residual feruloyl ester-degrading activity is retained in the $\Delta faeA$ mutant, probably from

MGG_10040 and/or *MGG_07653*, (ii) *MGG_10040* may encode a different ferulic acid esterase in *M. grisea*, independent of MgFaeA. It is suggested that the different feruloyl esterases from *A. niger* have complementary functions in the degradation of cell wall polysaccharides (de Vries & Visser 2001). Therefore, it is possible that any of the feruloyl esterases, other than MgFaeA, is required for pathogenicity, or a member of the feruloyl esterases with loss of function is complemented by the others.

Ferulic acid esterases from various microbial sources were intensively investigated, but most of these studies focused on the potential industrial application of feruloyl esterase, such as in food and feed processing or paper pulping (Liu et al. 2006). Our study attempts to elucidate the role of ferulic acid esterases in microbial pathogenesis, because ferulic acids are important structural components in providing cell wall integrity. To examine the role of MgFaeA in pathogenic development, we investigated *MgFaeA* expression during rice infection. RT-PCR analysis showed low levels of expression during early infection (24 hpi to 96 hpi) and abundant *MgFaeA* mRNA at later stages of lesion expansion (168 hpi), which may implicate the function of MgFaeA in the development of infection hyphae and the proliferation of fungus inside the plant cells. In addition, MgFaeA, working with other inhibiting protein, may also be indirectly involved in the fungus-plant interaction by producing structure-specific oligosaccharide fragments that are recognized by the plant host as elicitor signal molecules (Darvill et al. 1992; Ham et al. 1997). Our growing collection of purified MgFaeA protein, as well as the knockout mutant, allows us to continue the possible investigations.

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Functional Analysis of Two Laccase Genes in *Magnaporthe grisea*

Xin Chen, Wende Liu, Chuanzhi Zhao, Shuji Liu, Minoo Razee, Guo-dong Lu and Zonghua Wang

Abstract Laccase is found to be involved in pathogenicity of *Cryphonectria parasitica* and *Cryptococcus neoformans*. In this report we demonstrate that laccase is not necessary for pathogenicity in *Magnaporthe grisea*, which might be due to functional redundancy in some or all of the laccase genes. The major laccase activity in *M. grisea* is not encoded by either of the *MGG_00551.5* and *MGG_02876.5* genes, because targeted deletion of each gene shows only a slight decrease in laccase activity compared to wild-type strains. The *MGG_00551.5* and *MGG_02876.5* mutants share the same growth rate, conidiation and pathogenicity as wild-type strains. Taken together, our findings provide evidence that these genes are not essential for the differentiation and development of *M. grisea*.

Keywords Gene knockout · Laccase gene · *Magnaporthe grisea*

1 Introduction

Rice is a staple food resource for many people in the world, especially in many Asian countries. However rice blast has been a constant threat to the world's food supply (Sesma and Osbourn 2004; Ou 1985). The causal agent, *Magnaporthe grisea*, has been used for several decades as a model organism for understanding the mechanism underlying the plant host and fungal pathogen interaction (Valent and Chumley 1991; Talbot 2003; Dean et al. 2005). Molecular genetic analysis in several laboratories led to the identification of a number of *M. grisea* genes whose products contribute to the pathogenicity of the fungus (Odenbach et al. 2007).

It has been reported previously that laccase might play a role in virulence in *Cryphonectria parasitica* (Choi et al. 1992). Previous research on *Cryptococcus neoformans*' parasitism also found that laccase is related to its pathogenicity

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(Liu et al. 1999; Nosanchuk et al. 2000; Zhu et al. 2003). Therefore, we considered that laccase might also be an important pathogen virulence factor in *M. grisea*. According to annotation of the *M. grisea* genome, there are 17 putative laccase genes; we will systematically characterize the function of these genes. In this paper, we report the results of characterizing two putative laccase genes.

2 Materials and Methods

2.1 Fungal Strains, Culture Conditions and DNA Analysis

Wild-type strains were kindly provided by Dr. J.R.Xu, Purdue University. Mutant strains of *M. grisea* are stored at the Fungal Functional Genome Research Center, Fujian Agriculture and Forestry University. *M. grisea* growth, maintenance, nucleic acid extraction and transformation were performed according to standard procedures (Talbot et al. 1993). Gel electrophoresis and restriction enzyme digestion were performed using standard procedures (Sambrook et al. 1989).

2.2 Gene Replacement Vector and Mutant

A 843-bp fragment upstream from gene *MGG_00551.5* was amplified with primer MGG_00551.5 AF and MGG_00551.5 AR (Table 2) and cloned between the *Kpn*I and *Eco*RI sites of pCX62 to produce a plasmid named 551-A (Zhao et al. 2004). A 863-bp fragment downstream from gene MGG_00551.5 was amplified with primers MGG_00551.5 BF and MGG_00551.5 BR and cloned between the *Bam*HI and *Xba*I sites in 551-A. The resulting *MGG_00551.5* gene replacement vector 551-AB, in which a 2.2-kb fragment of the *MGG_00551.5* gene was removed and replaced with a 1.4-kb hygromycin phosphotransferase gene cassette, was transformed into protoplasts of the wild-type strain Guy11 (Xu & Hamer, 1996; Park et al. 2006). Hygromycin-resistant transformants were screened by PCR with two pairs primers: MGG_00551.5 OF and MGG_00551.5 OR; MGG_00551.5 UA and H853.

A 2.4-kb fragment of the *MGG_02876.5* gene was removed and replaced by the hygromycin phosphotransferase gene. The gene replacement vector of *MGG_02876.5* was constructed and introduced into rice pathogenic strain Ku70, which was defective for a protein that functions in nonhomologous end-joining of double-stranded DNA breaks.

2.3 Measurement of Growth Rate and Conidiation Analysis

The diameter of colonies was measured after 7 d growth on CM agar medium. Conidia were harvested in 5 mL sterile water from 14-d-old rice polish agar cultures by scraping the surface with a metal spreader, filtered through Miracloth, and then counted using a hemocytometer.

2.4 Rice Infection Assay

Plant infection assays were performed by spraying seedlings of rice (*Oryza sativa*) cultivar CO39, with suspensions of *M. grisea* conidia at a concentration of 10^5 conidia mL^{-1} (Dixon et al. 1999; Bhambra et al. 2006).

2.5 Laccase Activity Assay

Laccase extracts were prepared from supernatants of mycelia growing in CM liquid medium for 3 d. ABTS was used as substrate for the quantitative analysis of laccase activity. The enzyme activity was determined in culture supernatants with respect to the rate of oxidation of 500 μmol-1 ABTS (2, 2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) in acetic acid-sodium acetate buffer at 30 °C. The enzyme activity was expressed by the absorbance change of substrate at 420 nm, measured every 3 min. The experiments were performed in triplicates and the standard deviation was lower than 10% of the mean.

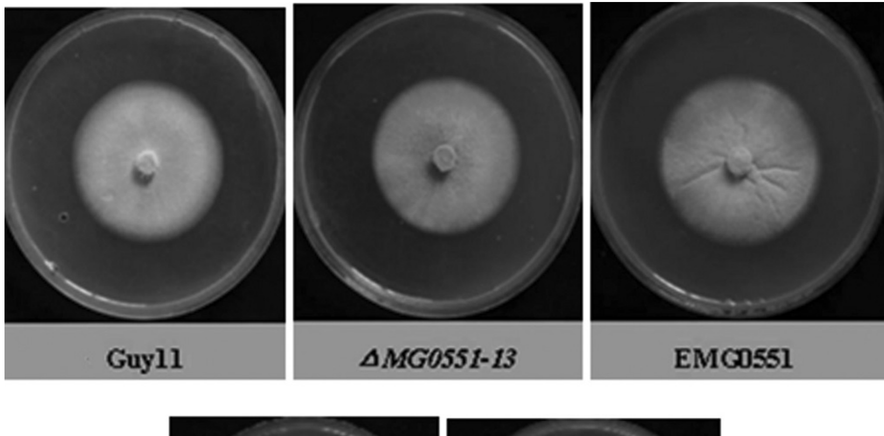
3 Results

In order to analyze the role of laccase genes in *M. grisea*, we first blasted the conserved amino acid sequence of the *Neurospora crassa* laccase against the *M. grisea* database; 17 putative laccase proteins were obtained. Gene *MGG_00551.5* encodes a 638 amino acid protein with similarity to the *N. crassa* laccase (28% amino acid identity). *M. grisea* also possesses another gene *MGG_02876.5* encoding a hypothetical laccase protein, which was closely related to the laccase protein of *N. crassa* (32% identity).

To investigate the function of the *MGG_00551.5* and *MGG_02876.5* genes in *M. grisea*, a targeted gene replacement vectors were constructed and introduced into rice pathogenic wild-type strains. Hygromycin-resistant deletion transformants were obtained and named *ΔMG0551-13* and *ΔMG2876*; an ectopic transformant was named EMG0551.

Growth rate test on CM agar medium revealed that each of the *ΔMG0551-13* and *ΔMG2876* mutants showed no obvious difference compared with wild-type strains, as shown in Fig. 1 and Table 1. To determine whether either gene encoded a major laccase protein, laccase activity was assayed. Both of the *ΔMG0551-13* and *ΔMG2876* mutants showed only a small decrease in their laccase activity level compared with wild-type strains, as shown in Fig. 2.

A detailed examination of virulence-associated functions was undertaken. We found that conidiogenesis in the *ΔMG0551-13* and *ΔMG2876* mutants was similar to that in wild-type strains (Table 1). The mutant spores germinated normally and developed appressoria (data not shown). The role of gene *MGG_00551.5* and *MGG_02876.5* in rice blast disease was investigated by inoculating seedlings of



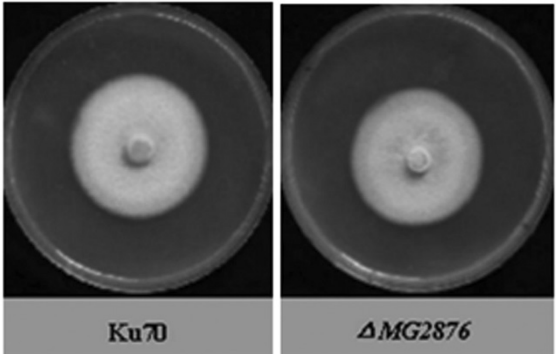


Fig. 1 Colonies of wild types, an ectopic transformant EMG0551-13 and mutant strains ΔMG0551-13 and ΔMG2876 growing on CM agar plates

Table 1 Growth rate and conidiation of ΔMG0551-13, ΔMG2876 mutants

Strain	Growth rate (mm/day)[a]	Conidiation ($\times 10^4$ conidia/plate)
Guy11	6.86 ± 0.11	30.58 ± 5.06
ΔMG0551-13	6.86 ± 0.13	27.32 ± 4.33
EMG0551	6.57 ± 0.15	25.25 ± 3.84
Ku70	6.50 ± 0.53	27.91 ± 3.02
ΔMG2876	6.48 ± 0.06	32.04 ± 0.52

[a] Growth rate was measured on CM agar plates after 7 days. Average growth rates and standard errors (mean ± standard errors) were calculated from at least three independent measurements.

the blast-susceptible rice cultivar CO39 with the *ΔMG0551-13* and *ΔMG2876* mutants. All mutants have the ability to cause rice blast disease in a similar way to the wild-type strains (Fig. 3). We therefore conclude that the *MGG_00551.5* and *MGG_02876.5* genes are not individually essential for development of rice blast disease.

Table 2 PCR primers used in this study

Name	Sequence (5′ → 3′)
MGG_00551.5AF	GGGGTACCCCTGCGTTGTTGTAATCG
MGG_00551.5 AR	CATCCAATCCAGTTGTCAGT
MGG_00551.5 BF	CGGGATCCATTCGTGCTGCTCGGGCTAC
MGG_00551.5 BR	GCTCTAGATGGGGATCTTGGATGATTGG
MGG_00551.5 OF	TGAGGGTCAGGCTATGGA
MGG_00551.5 OR	TGGAGGTGAAACGGATGG
MGG_00551.5 UA	GATGTGGGAGTTGGAGGTG
MGG_02876.5 AF	GGGGTACCTTGAAAGGTGGTTTGGTG
MGG_02876.5 AR	CGGAATTCGAAGGTAGGCGAGGACGA
MGG_02876.5 BF	GCTCTAGATTCTCCATCTTCGCTTCT
MGG_02876.5 BR	CGGAGCTCCTGCTCATCACTTCCACC
MGG_02876.5 OF	TGGTATCATTCGCACTACTCAG
MGG_02876.5 OR	GATCTCGGCAACAAACTCC
MGG_02876.5 UA	GTGATTTCGGTTCGGTTAC
H853	GACAGACGTCGCGGTGAGTT

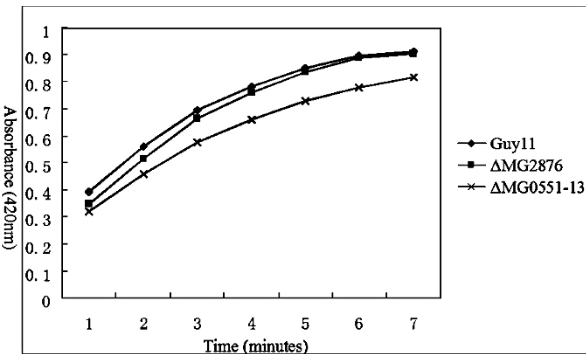


Fig. 2 The laccase activities of the ΔMG0551-13 and ΔMG2876 mutants compared to wild type strain Guy11

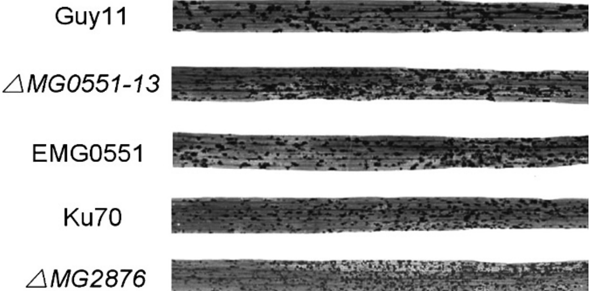


Fig. 3 The pathogenicity of ΔMG0551-13 and ΔMG2876 strains on rice. Two-week-old CO39 rice seedlings were sprayed with conidia of the rice pathogenic strains (Guy11, Ku70), an ectopic transformant (EMG0551), the gene MGG_00551.5 deletion mutant (ΔMG0551-13), and the gene MGG_02876.5 deletion mutant (ΔMG2876). Symptoms are shown at 7 days post inoculation

4 Discussion

In this study, the targeted *ΔMG0551-13* and *ΔMG2876* deletion mutants showed no observable differences compared to wild-type strains, not in growth rate, conidiation nor pathogenicity. And the laccase activity level in the *ΔMG0551-13* and *ΔMG2876* deletion mutants was only slightly lower than wild-type activity, so we cannot conclude that these genes encode major laccase activity. We suppose it is possible that some or all of laccase genes have functional redundancy.

In *C. neoformans*, and *C. parasitica*, laccase has been shown to be necessary for melanization, which is vital for pathogenesis in *M. grisea* (Howard et al. 1991; Money & Howard, 1996; Howard & Valent, 1996; de Jong et al. 1997; Bhambra et al. 2006). The characterization of laccase genes is therefore likely to be important in defining the regulation of melanization, as well as in diverse functions in plant infection for *M. grisea*. Experiments to determine the roles of other putative laccase genes and their interactions are currently underway.

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The Significance of Nitrogen Regulation, Source and Availability on the Interaction Between Rice and Rice Blast

Nicole M. Donofrio, Thomas K. Mitchell and Ralph A. Dean

Abstract Nitrogen regulation in fungi is a tightly controlled process, equipping them with the ability to colonize various ecological niches when preferred nitrogen sources are not available. Genes and pathways controlling this process have been well-defined in model, non-pathogenic fungi such as *Aspergillus nidulans* and *Neurospora crassa*. Research is now beginning to elucidate the importance of nitrogen regulation in pathogenicity. In this review, we will explore the regulatory mechanism called nitrogen catabolite repression in fungi and links between this and pathogenicity. We will examine what is currently known regarding nitrogen regulation and plant pathogenic fungi, with a focus on *Magnaporthe oryzae*, the devastating rice blast pathogen. Transcriptional activators involved in nitrogen catabolite repression are examined and compared among pathogens. We will also outline our own recent results describing a subtilisin serine protease that not only strengthens evidence for a link between nitrogen starved conditions and pathogenicity, but also appears to control expression of an important pathogenicity-related gene, *MPG1*.

Keywords *Magnaporthe oryzae* · Nitrogen scavenging · Nitrogen catabolite repression · Nitrogen-induced gene expression · GATA factor · *SPM1*

1 Introduction

Whether fungal infection begins from saprophytic hyphae, hearty overwintering structures or wind-blown spores, once it does begin, the pathogen most likely enters a completely different nutritional environment. In order for the fungus to survive, it must be equipped to cope with this drastic change. Studies of pathogenic fungi inhabiting different micro-niches within the plant host have revealed a spectrum of effects that nitrogen, as well as other nutrients, has on fungal gene expression,

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growth and development and ultimately, pathogenicity (for excellent reviews of overall nutrient utilization and nitrogen requirements by plant pathogens, see Snoeijers et al. 2000 and Solomon et al. 2003). The review presented herein will primarily focus on the relationship between nitrogen and plant pathogenic fungi, with an emphasis on the rice blast pathogen *Magnaporthe oryzae*. Studies on *M. oryzae* have contributed immensely to the field of nutrient requirements for plant-infecting fungi and their role in pathogenicity.

2 Nitrogen Starvation in *Magnaporthe oryzae* – A Rich History

Gene induction by nutrient depletion has been and continues to be a point of interest, particularly for researchers who examine pathogenic interactions. How pathogens move successfully from a non-pathogenic environment to entrance and colonization of a host depends on their own food reserves, as well as what the new surroundings have to offer, and their ability to take advantage of it.

In 1993, not long after *M. oryzae* had been developed as a 'model' plant pathogen for genetic studies by Barbara Valent and colleagues, a gene was discovered in a screen for cDNA expression during *in planta* growth. This particular gene, *MPG1*, turned out to be strongly expressed in the fungal penetration structure, or appressorium, during lesion development (late stage invasive growth) and during starvation for either nitrogen or glucose (Talbot et al. 1993). Shortly thereafter, two loci called *NPR1* and *NPR2* were identified and found to be involved in regulating starvation-induced gene expression (Lau and Hamer 1996). While these two loci were never cloned, this body of work led the authors to speculate that nitrogen possibly acted as an environmental cue for expression of pathogenicity genes. They tested this hypothesis by using nitrogen and carbon-starvation conditions to look at transcript expression; overall, nitrogen starvation induced the greatest number of transcripts. Further, the authors demonstrated that culture filtrates isolated from nitrogen starved mycelia were able to cause senescence when infiltrated into plant leaves, and that this senescence-causing capability was severely reduced in the *npr1* or *npr2* mutants, or when the fungus was deficient in a gene involved in turning on nitrogen scavenging pathways, *NUT1* (Talbot et al. 1997; Froeliger and Carpenter 1996). Together, these data suggested that nitrogen starvation not only mimics plant conditions, but also triggers the expression of genes required for successful infection.

3 Fungal Regulation of Nitrogen Catabolite Repression – Clever Control

NUT1, which stands for *n*itrogen *ut*ilization, was first identified by Froeliger and Carpenter in 1996. The gene was isolated and cloned using a reverse genetic approach based upon the sequence of a known nitrogen regulatory gene from the

related ascomycete, *Neurospora crassa*. The *N. crassa* gene, *nit-2*, is a zinc-finger DNA-binding gene that regulates expression of genes involved in nitrogen scavenging when preferred nitrogen sources, such as ammonium and glutamine, are limited or absent (Fu and Marzluf 1990a; Marzluf 1997). When only secondary nitrogen sources are present, this specific class of Zn-finger proteins receive this environmental cue and activate transcription of the many genes involved in nitrogen scavenging and utilization. Likewise, when preferred nitrogen sources are present, a transcriptional repressor called *nmr* in *N. crassa*, binds to the Zn-finger protein, inhibiting its binding to promoters of nitrogen scavenging genes and blocking their transcription (Pan et al. 1997; Fig. 1). In this manner, filamentous fungi and yeast achieve an exquisite level of genetic regulation in response to nitrogen source in a process referred to as nitrogen catabolite repression, or NCR.

Much of what is known today about NCR in pathogenic fungi stems from work on yeasts and the non-pathogenic model organisms, *N. crassa* and *Aspergillus nidulans*, in which this process has been well-characterized (reviewed in Marzluf 1997). Transcriptional activators function through their DNA binding domain, which contains the core consensus sequence GATA. They are therefore referred to as "GATA factors", and the promoter regions where they bind are "HGATAR" domains (reviewed in Marzluf 1997). Classical representatives of GATA factors are NIT-2 and AREA from *N. crassa* and *A. nidulans*, respectively (Fu and Marzluf 1990a; Crawford and Arst, 1993). Targeted deletion of these genes led to growth defects stemming from the mutant fungus' inability to utilize a vast array of nitrogen sources (Caddick et al. 1986; Fu and Marzluf 1990b). GATA factors that regulate nitrogen-related genes are conserved at the amino acid level among filamentous fungi (Table 1), with the most similar proteins to *M. oryzae* NUT1 being NIT-2 from *N. crassa* and CLNR1 from the bean anthracnose pathogen, *Colletotrichum lindemuthianum*.

Several GATA factors also show functional conservation through complementation of genetic mutations; *nit-2* can compensate for the defects in an *areA* mutant, restoring its ability to grow normally on various nitrogen substrates (Davis and Hynes 1987). A more recent study showed complementation of an *areA* mutant by the homologous GATA factor, *Nrf1*, from the tomato pathogen *Cladosporium fulvum* (Perez-Garcia et al. 2001).

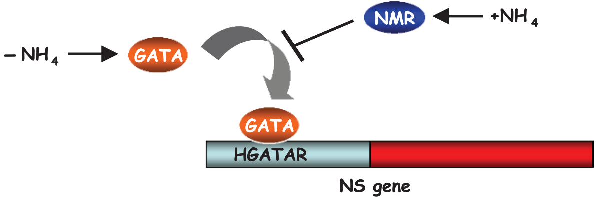

Fig. 1 Nitrogen catabolite repression model. When a preferred nitrogen source such as ammonium (NH_4) is absent, GATA proteins are activated and bind to the corresponding promoter regions (*light gray bar*) of genes involved in nitrogen scavenging (NS gene). Conversely, when a preferred nitrogen source is present, binding of the GATA factor to promoter regions is inhibited by the transcriptional repressor, NMR

Table 1 Amino acid similarity of the *Magnaporthe oryzae* NUT1 protein to nitrogen regulatory proteins

Gene	E-value	Identity	Similarity	Organism
CLNR1	0.0	497/1009 (49%)	622/1009 (61%)	*Colletotrichum lindemuthianum*
AreA	0.0	474/1017 (46%)	616/1017 (60%)	*Giberella fujikuroi*
NIT2	0.0	461/950 (48%)	590/950 (62%)	*Neurospora crassa*
FNR1	0.0	447/961 (46%)	586/961 (60%)	*Fusarium oxysporum f. sp. lycopersici*
NRR1	8e-155	444/993 (44%)	579/993 (58%)	*Metarhizium anisopliae*
NRF1	7e-103	355/946 (37%)	484/956 (50%)	*Cladosporium fulvum*
AREA	1e-82	325/888 (36%)	440/888 (49%)	*Aspergillus nidulans*
AREA	9e-80	315/843 (37%)	419/843 (49%)	*Aspergillus fumigatus*
DNR1	3e-70	234/654 (35%)	337/654 (51%)	*Microsporum canis*

Considering all the genetic and functional homology among GATA factors from filamentous fungi, it is logical that researchers would shift focus to these proteins to learn more about how fungi cope with nitrogen stress, and whether this relates to pathogenicity. At least seven GATA factors have been identified and characterized from pathogenic fungi, and these are shown in Table 2. There is currently no clear correlation emerging between requirements of GATA factors in pathogenicity, and either lifestyle, or host micro-niche, of the pathogen. Both *M. oryzae* and the bean anthracnose pathogen *Colletotrichum lindemuthianum* are considered hemi-biotrophs, yet *NUT1* is not required for full pathogenesis, while *CLNR1* is not only required for full virulence, but also for switching from a biotrophic to a necrotrophic lifestyle. In their recent review, Divon and Fluhr (2007) make the interesting point that for hemi-biotrophs, the effect of AreA-like GATA factors on pathogenicity might depend upon how long the pathogen spends in the biotrophic phase of its life; *C. fulvum* is apoplastically located for the entirety of its infection cycle and likewise, *C. lindemuthianum* spends more time in a biotrophic phase, in comparison to *M. oryzae*. Accordingly, *Nrf1* and *CLNR1* play a role in virulence, while *NUT1* does not.

While *NUT1* itself is not essential for pathogenicity, it has a demonstrable role in regulating expression of genes involved in this process. *MPG1*, for example,

Table 2 Roles of nitrogen regulatory proteins in pathogenesis

Gene	Organism	Pathogenesis	Reference
NUT1	*M. oryzae*	no effect	(Froeliger and Carpenter, 1996)
AREA	*G. fujikuroi*	unknown	(Tudzynski et al., 1999)
CLNR1	*C. lindemuthianum*	less virulent/no switch	(Pellier et al., 2003)
FNR1	*F. oxysporum*	less virulent	(Divon et al., 2006)
NRR1	*M. anisopliae*	unknown	(Screen et al., 1998)
NRF1	*C. fulvum*	reduced virulence	(Perez-Garcia et al., 2001)
AREA	*A. fumigatus*	slightly less virulent	(Hensel et al., 1998)
DNR1	*M. canis*	unknown[1]	(Yamada et al., 2006)

[1] the *M. canis dnr1* mutant was shown to have deficient growth on media containing keratin, which is an important consideration as this is a skin pathogen of animals.

relies on *NUT1* for full expression (Lau and Hamer 1996). Further, we can learn which genes are potentially involved in NUT1 binding and derepression of NCR during starvation conditions, by examining promoter motifs. A beacon for GATA factor binding is the HGATAR motif. Genes that bind and are therefore activated by GATA factors such as AREA or NIT-2, contain at least one, and often more than one, HGATAR motif where H is A, T or C and R is A or G (Chiang and Marzluf 1994; Gomez et al., 2003). We performed a global transcription profiling of *M. oryzae* mycelia that had been shifted from media containing a nitrogen source to a nitrogen-free media, and compared this sample to a non-starved culture. Our experiment yielded 520 and 345 genes with increased and decreased expression versus the non-starved culture, respectively. Our interest in how NCR functioned in a plant pathogen prompted us to mine the promoters of both gene sets for presence of HGATAR motifs. We hypothesized that genes with starvation-induced expression would contain a higher number of HGATAR promoter motifs versus a down-regulated gene set. We analyzed predicted promoter regions from 50 genes with the most dramatic fold changes in each set, and included a ‘background’ set represented by a random sampling of 50 genes from the complete, unfiltered data. Our results confirmed our hypothesis; almost twice as many HGATAR motifs were present in the up-regulated promoter group as compared to either the down-regulated or the background set, and the latter two sets did not significantly differ from each other (Fig. 2). Further, 70% of promoters from the up-regulated group

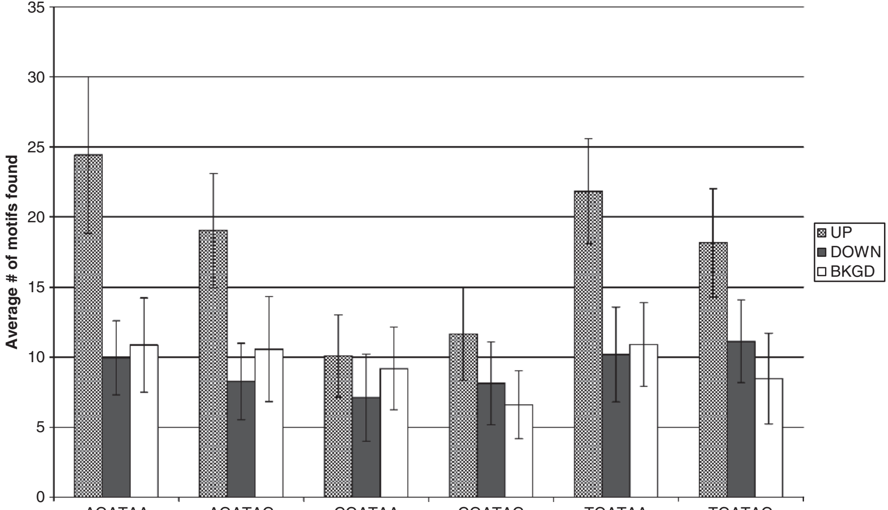

Fig. 2 Graph shows the presence of HGATAR motifs in promoters taken from the 50 most up-regulated genes, the 50 most down-regulated genes and 50 genes generated from a random sampling. *Gray bars* indicate the up-regulated group, black is down and white shows the background group. The *x*-axis is motif type, while the *y*-axis shows average number of each motif type found. In all but two types of HGATAR motifs, the up-regulated group had more motifs per promoter, and more promoters containing these motifs

contained more than one HGATAR motif, which has a demonstrable effect on GATA binding efficiency in *A. nidulans* (Ravagnani et al. 1997).

4 Nitrogen Starvation in the 'OMICS' Century – New Information from New Approaches

Scientists have taken advantage of genomic approaches to identify genes involved in numerous physiological pathways and processes including nitrogen starvation, which is thought to mimic the plant pathogen's natural host environment. A starvation-induced cDNA library was made from the tomato pathogen, *Cladosporium fulvum*, and five transcripts induced during starvation were also induced *in planta* (Coleman et al. 1997). Transcriptional profiling in *Fusarium graminearum*, causal agent of head blight of wheat, included a library derived from nitrogen starved cultures that yielded the highest number of genes from any of the three libraries with similarity to known pathogenicity genes in other fungi (Trail et al. 2003). Two genes from this group were similar to *M. oryzae* genes involved in appressorial formation; *MAC1*, an adenylate cyclase gene involved in cAMP-directed signaling, and *PTH2*, a gene with peroxisomal carnitine acetyltransferase activity (Choi and Dean 1997; Bhambra et al. 2006). Together, this work demonstrates the importance of nitrogen starvation in identifying genes involved in pathogenicity.

Likewise, the aforementioned transcriptional profiling of nitrogen-starved *M. oryzae* mycelia not only identified known pathogenicity genes including *MPG1*, the integral membrane protein *PTH11*, tetrahydroxynapthalene reductase (involved in melanization), alternative oxidase and the neutral trehalase *NTH1* (DeZwaan et al. 1999; Avila-Adame and Koller 2002; Foster et al. 2003), but at least one new gene involved in virulence (Donofrio et al. 2006). *SPM1* is a subtilisin serine protease originally cloned in 2002 (Fukiya et al. 2002), but at the time its function was only predicted. It re-appeared in the nitrogen starvation profiling experiment, showing a significant fold increase of expression over mycelia in the presence of nitrogen. A targeted deletion of this gene revealed its multiple roles in spore production, appressorial formation and invasive growth, as all three were comprised in the *spm1* mutant (Donofrio et al. 2006). *spm1* was also deficient in its ability to utilize various secondary nitrogen sources, a phenotype reminiscent of the nitrogen regulatory loci mutants *npr1* and *npr2* (data not shown). While this gene did not turn out to account for either of those loci (Donofrio et al. unpublished results), this finding did prompt us to examine expression of the hydrophobin gene, *MPG1*. As mentioned previously, this gene's expression is compromised in the *npr1* and *npr2* mutants, and its expression is also regulated by nitrogen (Lau and Hamer, 1996; Talbot et al. 1997). *MPG1* expression was tested in the *spm1* deletion mutants as well as wild type and an *spm1* ectopic; results revealed that, as previously noted, *MPG1* expression is regulated by nitrogen, and in addition, by the *SPM1* gene (Fig. 3). This finding could account, at least in part, for the pathogenicity-related phenotypes observed in

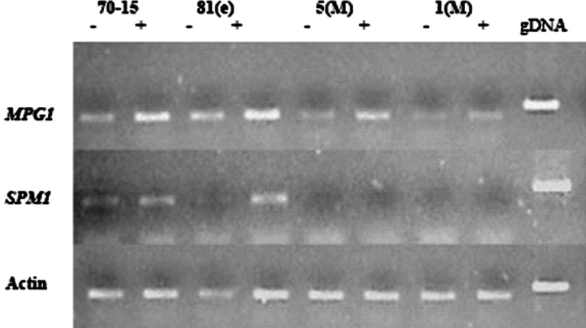

Fig. 3 SPM1 expression of the pathogenicity-related hydrophobin MPG1. Reverse-transcription PCR analysis shows nitrogen-based gene regulation patterns. 70–15: wild type; 81(e): spm1 ectopic; 5(M): spm1 mutant; 1(M): spm1 mutant; gDNA: genomic DNA. Genomic DNA was used to show that primers were amplifying the transcripts, and products were not due to genomic contamination. + and − indicates the presence or absence of nitrogen, respectively. Actin was used as a house-keeping gene

the *spm1* mutant. Further analysis of *SPM1* will reveal the nature of its true role in pathogenesis and nitrogen starvation.

Genomic profiling of nitrogen starvation also provided insight into how this NCR functions in the rice blast pathogen. We filtered induced genes based upon their predicted functions and found that 39 had similarity to genes from other organisms with known roles in nitrogen scavenging. One of these genes is a putative methylammonium transporter, and is noteworthy due to the fact that in the corn pathogen *Ustilago maydis*, it plays a role in switching from a pseudohyphal to an invasive, filamentous growth during low nitrogen conditions. When this transporter, *UMP2*, is deleted, *U. maydis* cannot effectively switch morphologies (Smith et al. 2003). The *M. oryzae* homolog shares 70% amino acid similarity to *UMP2* in *U. maydis* and awaits functional characterization to determine its role both in nitrogen scavenging as well as growth morphology. Taken together, genomic studies that include a nitrogen starvation sample not only show this condition's utility to identify known and new virulence determinants, but also to better understand the complex and intertwined regulation of nutrient acquisition and pathogenicity.

5 Consequences of Nitrogen on Plant Disease – From the Lab to the Field

When considering the effects of nitrogen starvation on plant pathogens, one must also consider the plant. Several studies have aimed to "peer inside" the plant cell in order to determine the type and role of nitrogen sources present during pathogen attack. In 1997, Talbot and colleagues collected culture filtrates from nitrogen starved *M. oryzae* and found that they contained senescence-inducing compounds

when tested on plants. The induction of such compounds indicated that a nitrogen starved condition mimicked that of the plant environment. In 1999, Snoeijers et al. discovered that the avirulence gene, *AVR9*, from *C. fulvum* was induced by nitrogen starved conditions and furthermore that this gene contained HGATAR promoter motifs (Snoeijers et al. 1999). This was followed by a study in 2001 that sought to identify the components of apoplastic fluids from tomato infected with *C. fulvum*; results showed a high amount of the nitrogen source, γ-alphabutyric acid (GABA) (Solomon and Oliver 2001). While this suggested *C. fulvum* might not be in a nitrogen-limited environment upon first entering the host, an alternate hypothesis is that the fungus manipulated the host to re-route nitrogen sources to the infection site.

Recently, this hypothesis was tested in studies involving nitrogen content and host gene expression of plants infected with viruses, bacteria or fungi. Two genes involved in nitrogen re-mobilization, glutamine synthetase (*GS1*) and glutamate dehydrogenase (*GDH*) from tobacco were analyzed for expression during infection with three *P. syringae* strains; compatible, incompatible with a hypersensitive response (HR) or incompatible without an HR (Pageau et al. 2006). Results indicated that while *GDH*, a gene involved in nitrogen transport, is induced regardless of which bacterial strain is infecting, *GS1*'s expression was mainly induced by the incompatible bacteria, implicating a role for this gene in defense. What that role is remains unclear, however a recent study on nitrogen content and *GS1* expression during infection of bean by *C. lindemuthianum*, revealed an accumulation of glutamine, a preferred fungal nitrogen source, and *GS1* transcript levels, in infected leaves (Tavernier et al. 2007). The authors utilized three strains in their studies; wild type, a mutant defective in penetration and a mutant defective in switching from a biotrophic to necrotrophic phase. While glutamine accumulated in leaves after inoculation with all three fungal strains, levels continued to rise in the wild type infection, reaching 33%. In the mutant-inoculated leaves, glutamine levels returned to that of control leaves (2%) by a later stage of infection. Interestingly, *GS1* expression matched that of two defense-related genes, *PAL* and *CHS*. Further investigation is required to determine whether the plant is being manipulated to produce and/or re-mobilize its nitrogen contents to the expanding infection court, or whether nitrogen is being shuttled away from the court in a metabolic defense program (Pageau et al. 2006).

With nitrogen being critical for a pathogen's survival success, it is no surprise that scientists would be concerned about the impact of nitrogen-based fertilizers on disease in the field. Since the 1930s, correlations have been drawn between the impacts of nitrogen fertilization and disease incidence of crops, and these earlier results are summarized in Snoeijers et al. (2000). More recent studies provide additional supporting evidence for these observations, along with an interesting mechanistic basis for the correlation. Changes to plants induced by higher amounts of nitrogen fertilizers applied in the field include increases in canopy and crop density, which fosters a favorable micro-climate for pathogen infection and spread (reviewed in Neumann et al. 2004). However, a study on yellow rust of wheat, caused by the pathogen *Puccinia striiformis* f. sp. *tritici*, challenged this point by applying nitrogen after canopy

development was complete; their results showed that disease severity still increased, and had little to do with canopy development (Neumann et al. 2004). Furthermore, they determined that the type of nitrogen applied was important to disease severity; while both ammonium and nitrate increased canopy size, only the latter contributed to disease severity, indicating that it was the specific nitrogen component in the host leaf that was an important factor in increased disease severity. Conversely, the type of nitrogen applied to strawberries showed no difference in disease severity of the anthracnose pathogen—both types of nitrogen increased disease (Nam et al. 2006).

The mechanism for this positive correlation between nitrogen fertilizers and enhanced disease was examined in red pine. Application of fertilizers increased disease caused by the canker and stem blight fungal pathogen (Blodgett et al. 2005). Experimental results demonstrated significant increases of foliar nitrogen and decreases of lignin and phenolic compounds, which are known to be involved in defense in red pine, upon fertilization. In rice, blast disease incidence and severity increased across a range of differentially susceptible cultivars (except in the most resistant one) upon a higher- than-average application of nitrogen (Long et al. 2000). The authors also observed that, contrary to observations on other crop plants, rice matured more slowly under the higher nitrogen regime, leaving its window of susceptibility open for a longer period of time.

How does this information help reduce disease incidence in the field? There is a positive correlation between higher nitrogen application and disease severity/incidence in many crop species. Further, a growing body of evidence indicates that we should not only consider the effects of nitrogen fertilizers on plant maturation and canopy, but that we need to gain a better understanding of how 'feeding the plant', results ultimately in 'feeding the pathogen'.

6 Complex Networks – Nitrogen's Effect on Other Pathways and Processes in *M. oryzae*

It is becoming more evident that nitrogen regulation is intimately connected with other physiological processes, all leading to successful infection in the rice blast pathogen. Recent studies from Talbot and colleagues exemplify this concept. They examined the role of trehalose in *M. oryzae* infection and determined that while production of trehalose was necessary for appressorium formation by the trehalose-6-phosphate synthase gene *TPS1*, its subsequent breakdown was needed for invasive growth (Foster et al. 2003). A deeper foray into *TPS1* recently showed that along with this gene's role in production of trehalose and functional appressoria, it also had a hand in nitrogen metabolism (Wilson et al. 2007). Previously, the authors had noted that *tps1* mutants could utilize nitrite and ammonium as sole nitrogen sources, depending upon the carbon source present; however, it was unable to grow on nitrate, regardless of the carbon source available. This led the authors to question whether the absence of TPS1 resulted in a breakdown of nitrogen catabolite

repression. Indeed they found that transcript levels of the negative regulator of NCR, *NMR1*, were dramatically higher in the *tps1* mutant versus the wild type strain when grown on either nitrate or ammonium. *NMR1* is equivalent to *nmrA* in *A. nidulans*, the negative regulator of *AreA* in the presence of primary nitrogen sources (Andrianopoulos et al. 1998). Based upon their results, the authors present a model of how both nitrogen and carbon sources are sensed by the fungal cell in independent processes, linked together by the *TPS1* gene. Their study reveals the tightly regulated and complex pathways of carbon and nitrogen source utilization and their contribution, through the *TPS1* gene, to pathogenicity (Wilson et al. 2007).

7 Conclusions and Future Prospects – What's on the Horizon for Nitrogen Regulation and Pathogenicity in *M. oryzae*?

Over a decade's worth of study has indicated that nitrogen utilization is important to pathogenicity and successful infection in *M. oryzae*. Furthermore, it is entirely plausible that this fungus may find itself in a nitrogen starved environment upon entering a plant (Talbot et al. 1997). Recent data on structures called woronin bodies provide strong evidence for this hypothesis. Woronin bodies are dense organelles derived from peroxisomes and when *M. oryzae* mutants lack the gene for woronin body production, the fungus cannot produce functional appressoria, or grow invasively *in planta*. Interestingly, mutants are also unable to survive nitrogen starvation *in vitro*, indicating that the invasive growth defect is likely due to the fungus' inability to deal with a nitrogen-limited environment in the plant (Soundararajan et al. 2004).

Many challenges remain in this field, and perhaps one of the grandest in scope is how to apply insight from laboratory work to the field. An overwhelming body of evidence points to excess nitrogen fertilization leading to increased disease severity in the field; and yet at the same time, fertilization promotes hearty and rigorous growth, in some cases coaxing the plant to achieve maturity more quickly, hence becoming more immune to plant diseases (Blodgett et al. 2005). One pertinent question is, if indeed we are 'feeding the fungus' by application of nitrogen sources to the plant as some work has suggested, how is the fungus sensing this source and using it to it's advantage? An answer to this question might come in part from studying nitrogen sensors and receptors, such as the methylammonium transporters, *MEPa* and *MEAa* homologs, both of which we found to be increased in expression in the rice blast pathogen in response to nitrogen starvation (Donofrio et al. 2006). If these transporters function in *M. oryzae* as they do in other fungi, then it may be possible to someday design a fungicide that specifically targets and inhibits them, thus inhibiting the uptake of nitrogen from a plant host.

The role of proteases in nitrogen utilization and their link to pathogenicity in *M. oryzae* poses another interesting challenge. *SPM1* is necessary for full virulence, and the defect appears to be quantitative in nature with regard to production of spores and appressoria, as well as invasive growth (Donofrio et al. 2006). But what, exactly, is the underlying mechanism of this defect and how does the fact that it is under

nitrogen regulation, fit in? In the fungal insect pathogen *Metarhizium anisopliae*, a subtilisin protease, *PR-1*, functions by dissolving the insect cuticle, making room for penetrating hyphae and providing a food source for further growth (see StLeger et al., 1996). It is also under control of nitrogen catabolite repression, similar to *SPM1* from *M. oryzae* and shares 68% amino acid similarity with *SPM1*. Whether *SPM1* plays a role in plant cell wall degradation remains to be examined, as well as whether nitrogen impacts this process. Another recently described gene in *M. oryzae*, *snodprot1*, was shown to be secreted by the pathogen and involved in invasive growth during infection. Transcript levels of *snodprot1* increase upon nitrogen starvation, however how these two elements are connected, remain to be determined (Jeong et al. 2007).

For more than a decade, *M. oryzae* has proven itself a model system in which to study nitrogen and its role in pathogenicity. As the pace and technology of scientific discovery increases, we will be able to further discern what this fungus is faced with when it enters a susceptible plant host, and how it makes the most of its surroundings to be one of the most successful and devastating fungal plant pathogens worldwide.

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Protein Chips and Chromatin Immunoprecipitation – Emerging Technologies to Study Macromolecule Interactions in *M. grisea*

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Abstract In the post genome era, a major challenge is to understand the transcriptional control of each gene and the network of interactions control growth, development, and differentiation. The genome sequence of many fungi is now available, as are growing data sets of large-scale transcriptional studies. Microarray analysis reveals that specific sets of genes are coordinately regulated, presumably via the action of largely uncharacterized transcription factors. Those studies provide a clue to the function of co-regulated genes as well a means to categorize groups of genes. However, the ultimate objective is to be able to model the underlying pattern of associations that provides the transcriptional control. The finished genome assembly has allowed us to annotate a relatively complete list of proteins associated with transcription initiation and/or regulation. Using emerging technologies initially designed to study humans and model systems like yeast, we can now identify protein interactions that lead to transcription factor regulation as well as reveal the specific nucleotide binding sites for any DNA binding protein. Specifically, using protein chips and ChIP-chip studies, we can begin to examine transcriptional circuits that regulate the infection and development processes in this important plant pathogen.

Keywords Protein chip · ChIP-chip and Transcription factors

1 Introduction

The importance of *M. grisea* as a pathogen of rice, the food staple of over half the world's population, warrants extensive research activities aimed at understanding its growth, development, and pathology. Few other plant pathogens, fungal or other,

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have this impact on a global scale in terms of nutrition, livelihood, culture, and economic well-being (Dean 1997). While host plant resistance is the most economically viable and environmentally sound approach to control disease, this pathogen overcomes new resistance genes relatively rapidly to render some new rice varieties impotent. To design durable host defense strategies, a complete understanding is needed of how this pathogen recognizes its host and transfers environmental signals to the nucleus to set in motion the tightly orchestrated events enabling infection and host death. While knowledge of the core signal pathways is emerging, the key determining steps controlling environmental perception and cellular responses are as yet a mystery (Dean 1997). Specifically, we have little knowledge of the receptors used by the fungus to detect a suitable host, nor do we know what downstream factors specifically bind to and tightly regulate the expression of genes deployed during infection-related development and invasive growth *in planta*.

2 The *M. grisea* Genome and Transcriptome

One of the most significant recent achievements for *M. grisea* was the completion of a draft sequence (Dean et al. 2005). Following the initial characterization of the $\sim$ 40 Mb genome and its 11,108 predicted genes, the work of finishing to close most of the remaining gaps was completed. Using information from the draft sequence, large-scale functional studies including extensive microarray studies were launched in 2001. That research effort generated a 22,000-element oligo-based microarray in collaboration with Agilent Technologies (G4137A) that contains the Broad Institute version 5 predicted gene set, plus additional predicted features from other gene models, as well as ESTs for genes not predicted. Using this array, transcription profiling experiments to identify genes expressed during infection, appressoria formation, growth under various nutritional stresses, and in mutated genome backgrounds were completed. Protocols used and microarray data can be accessed through NCBI GEO www.ncbi.nlm.gov/projects/geo/under accessions GSE2716 and GSE1945. Additional information on these studies can be found at MGOS, www.mgosdb.org. As anticipated, the results revealed that hundreds of genes are differentially expressed when any two conditions are compared. For example, 529–612 genes, representing $\sim$ 4% of the total predicted gene set are up-regulated during spore germination (Table 1). Similar numbers are down regulated. In addition, about 2 and 4% of genes are significantly differentially regulated in immature (7 hr) and mature (12 hr) appressoria, respectively, compared to spores germinated on a non-appressoria-inducing surface. Similar numbers of genes are differentially regulated during growth in carbon and nitrogen limited environments (Dean et al. 2005). Similar experiments to evaluate whole genome expression mutant backgrounds, including *pmk1* and *mst12*, have also been done (unpublished data). In sum, due to sheer numbers the ability to select candidate genes for additional studies remains challenging at best. New advanced methods need to be developed to complement transcriptional studies to more expertly direct research efforts.

Table 1 Differential gene expression during germination and appressorium formation

Condition	# genes 2 fold up	# genes 2 fold down
Appressorium inducing 7 hr vs Spore	588	652
Appressorium inducing 12 hr vs Spore	612	972
Appressorium non-inducing 7 hr vs Spore	603	493
Appressorium non-inducing 12 hr vs Spore	529	497
Appressorium inducing 12 hr vs 7 hr	321	187
Appressorium non-inducing 12 hr vs 7 hr	37	22
7 hr Appressorium inducing vs non-inducing	194	85
12 hr Appressorium inducing vs non-inducing	352	133

3 Key Transcription Factors Function Downstream of Important Signaling Pathways

Although there is growing knowledge of the core elements of the signaling pathways that regulate plant infection processes in many pathogens, our knowledge of what lies up and downstream is fragmentary at best. One efficient approach to further characterize genes regulated by these pathways is to identify the downstream transcription factors. Transcription factors, are generally defined as any protein, other than RNA polymerase, required to initiate or regulate transcription in eukaryotic cells. General factors required for transcription of all genes, participate in formation of the transcription-initiation complex near a start site (Latchman 2004). In addition, several thousand specific transcription factors have been thus far identified that share two characteristic features: a DNA binding domain that interacts with gene-specific regulatory sites, and a domain that exhibits transcriptional activation potential (see the TRANSFAC database, www.gene-regulation.com/pub/databases.html). Transcription factors may exhibit a specific combinatorial distribution in different cell types that helps to direct determination (choice of cell fate) and differentiation (synthesis of recognized cell-specific proteins). Characteristic features include Zn finger domains, homeodomains, b-zip (basic leucine zipper), helix-loop-helix (HLH), to name a few. Certain domains such as homeodomains are also associated with protein-protein interactions (Latchman 2004). Others such as HLH form homo- or heterodimers when binding to DNA. Two novel DNA binding domains are found extensively in fungi. IPR001138 is a cys-rich domain that requires Zn for binding and IRP007219 is found commonly in genes regulating metabolism and development. Many fungal transcription factors, including GAL4, contain both fungal specific domains (Hashimoto et al. 1983). The number of transcription factors estimated in eukaryotes varies considerably. *S. cerevisiae* contains in the range of 141–209 depending on the selection criteria, whereas Arabdopisis contains an estimated 1,827 which fall into 56 families (http://datf.cbi.pku.edu.cn/). Based on the draft genome sequence of *M. grisea*, we estimate there are $\sim$ 500 (excluding chromatin remodeling and related factors). The protocol used to annotate these is outlined in Fig. 1 and the most abundant domains as classified by InterPro categories are shown in Table 2.

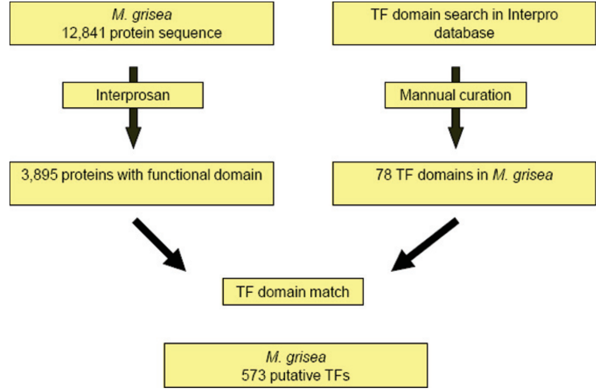

Fig. 1 Strategy for annotating transcription factors (TFs) based on the existence of DNA binding regulatory domains. InterPro domains that mapped to sequence-specific DNA-binding transcription factor GO categories such as GO:0003700 (Transcription factor activity), GO:0003702 (RNA polymerase III transcription factor activity), GO:0016563 (Transcriptional activator activity), and GO:0016563 (Transcriptional repressor activity), were identified. Interpro domains we also extracted by key word searching of "transcription factor", "DNA binding" and "regulatory protein" from IntroPro database. The results were manually inspected and the domains that are not directly involved in transcriptional regulation were eliminated. Next, the predicted protein sequences of *M. grisea* were analyzed with InterProScan program (verson 10.1), where the individual sequences are scanned against the protein signatures of the InterPro member databases including UniProt, *PROSITE*, Pfam, PRINTS, SUPERFAMILY, ProDom and SMART. Sequences containing the selected interPro domains were marked and manually curated

While transcription factors are typically activated post translationally, microarray studies revealed that some of them exhibit differential expression. Expression of 305 putative transcription factors during spore germination and appressorium formation can be detected. One hundred and twenty-eight gave normalized expression

Table 2 Summary of most abundant *M. grisea* transcription factor domains based on InterPro scan

Interpro ID	Interpro name	# of Domains
IPR001138	Fungal transcriptional regulatory protein, N-terminal	121
IPR007087	Zn-finger, C2H2 type	96
IPR007219	Fungal specific transcription factor	52
IPR001005	Myb, DNA-binding	35
IPR004827	Basic-leucine zipper (bZIP) transcription factor	21
IPR001965	Zn-finger-like, PHD finger	14
IPR011616	bZIP transcription factor, bZIP_1	12
IPR002409	Aflatoxin biosynthesis regulatory protein	12
IPR011700	Basic leucine zipper	11
IPR001789	Response regulator receiver	11
IPR000910	HMG1/2 (high mobility group) box	10
IPR003347	Transcription factor jumonji, jmjC	9
IPR001092	Basic helix-loop-helix dimerisation region bHLH	9
IPR000679	Zn-finger, GATA type	8
IPR002197	Helix-turn-helix, Fis-type	7
IPR001356	Homeobox	6

values more than twice threshold level and 12 were differentially expressed during appressorium formation.

To date, few transcription factors have been characterized in phytopathogenic fungi. In *S. cerevisiae*, Ste12 is a transcription factor functioning downstream from the Fus3/Kss1 MAP kinases. Ste12 is essential for mating and also involved in filamentation. In *M. grisea*, however, *MST12* (Ste12 homolog) is not essential for appressorium formation but required for appressorial penetration and invasive growth (Park et al. 2002; 2004). The C-terminal portion of MST12 weakly interacts with PMK1 in yeast two-hybrid assays (Park et al. 2002). In co-immunoprecipitation experiments, PMK1 associates with MST12 in proteins isolated from mycelia (Xu, unpublished). Appressoria formed by *mst12* mutants are normal in morphology and turgor generation but fail to form penetration pegs, probably due to cytoskeleton defects in mature appressoria (Park et al. 2004). Therefore, transcription factor(s) other than MST12 must exist in *M. grisea* and function downstream from PMK1. In *C. lagenarium*, CMK1 (the PMK1 homolog) also is dispensable for appressorium formation but required for penetration and pathogenic growth (Tsuji et al. 2003). *M. grisea* gene (*Pth*12) recovered from a REMI screen, encodes a homeodomain-containing transcription factor (Jim Sweigard unpublished data). The *pth*12 mutant produces rare, small appressoria and are unable to infect. It has no obvious defects in growth and conidiation, and is also fully fertile. In *Ustilago maydis*, the transcription factor PRF1 was originally identified as the key pheromone response factor regulating mating and sexual development (Hartmann et al. 1999). Further characterizations indicate that PRF1 functions downstream of both the cAMP-dependent protein kinase A and MAPK KPP2 in *U. maydis*. The phosphorylation of PRF1 by PKA and KPP2 have different effects on transcriptional responses of pheromone induced cells (Muller et al. 1999; Kaffarnik et al. 2003). These data indicate that PRF1 is regulated by both the cAMP signaling and MAP kinase pathways and it plays critical roles in both mating and plant infection.

4 Protein Microarrays

While there is a significant body of knowledge about the core pathways in fungal pathogens as previously described, there is little or no information regarding the transcription factors they interact with nor the sets of genes these transcription factors specifically regulate. With the advances in protein microarrays, we now have a means to fill this void in a robust, high-throughput fashion. Protein microarrays, by definition, contain a defined set of proteins spotted and analyzed in parallel at a high density (Zhu et al. 2003). The advantage of protein microarray based experiments lies in the ability to rapidly screen large numbers of proteins simultaneously for biochemical activities, protein-substrate binding activities (e.g. protein-protein, protein-DNA, protein-lipid, and protein-drug interactions), and posttranslational modifications. Other advantages include low reagent consumption, controlled *in vitro* assay conditions, high sensitivity, automation, and direct target identification

(Zhu et al. 2003). Furthermore, protein chips have the unique advantage in that they can be used to analyze protein/protein-complex interactions (Zhu and Snyder 2002).

In 2001, Zhu (co-PD) demonstrated that a whole-proteome microarray for eukaryotes could be made and applied for functional assays (Zhu et al. 2001). Using a yeast recombination cloning strategy, the team cloned 5,800 ORFs (94%) of the yeast genome as N-terminal fusions into a yeast expression vector under the control of an inducible promoter. To purify 6,000 proteins from yeast cells, a high-throughput protein purification protocol was developed. Every step is done in a 96-well format from cell culture to protein elution. To attach proteins to a solid substrate, they used both covalent and affinity-based strategies to orientate proteins uniformly away from the surface where they are more likely to remain in their native conformation and activity. Using the yeast proteome microarrays, they found that the assay for protein-protein interactions is extremely powerful as they could identify almost every potential binding partner of a given probe in a single experiment. For example, when the yeast homologues of calmodulin and 14-3-3 proteins were used to probe the yeast proteome chips, they identified 33 and ~ 140 potential targets, respectively. In the calmodulin binding experiments, they not only identified six known targets, but also revealed 27 additional candidates. Using an unbiased bioinformatics approach, 19 of these targets were found to share an IQ motif, whose sequence was extremely similar to the previously known IQ motif of calmodulin binding proteins. Since 14-3-3 proteins were known to bind to many phosphoproteins, co-immunoprecipitation (co-IP) experiments were done to confirm novel targets identified and validated more than a dozen new 14-3-3 binding partners (H. Zhu, unpublished data). This technology is now being adapted to map transcription factor targets for phosphorylation by specific kinases known to be involved in morphogenesis and pathogenesis by *M. grisea*.

5 Kinase Assays on Transcription Factor Protein Chips

The importance of protein phosphorylation to all facets of cell physiology can not be overstated and is reflected in the fact that protein kinase genes are highly conserved and found in a large fraction (~ 2%) of most eukaryotic genomes (Rubin et al. 2000; Manning et al. 2002a,b). Moreover, approximately 30% of cellular proteins are estimated to be phosphoproteins (Cohen 2000; Ficarro et al. 2002). Using protein chips, it is possible to globally identify kinase substrates by incubating the proteome arrays with cloned protein kinases and labeled ATP and identify phosphorylated proteins. Zhu and colleagues in Michael Snyder's laboratory at Yale University used this approach to identify potential substrates for 87 yeast protein kinases (Zhu and Snyder, unpublished). These kinase assays yielded a total of 4,192 phosphorylation events affecting 1,325 proteins. Results from this study are available at http:networks.gersteinlab.org/~xzhu. This dramatically increased the number of previously known kinase-substrate interactions of ~ 120. A distinct set of substrates was phosphorylated by each protein kinase, indicating that each

has a unique substrate recognition profile. Most (73%) substrates were recognized by fewer than three kinases, indicating a strong preference for particular kinases. When the substrate lists of closely related kinases were inspected, distinct sets of substrates were revealed for each kinase. For example, in the case of the three PKA homologs in yeast, Tpk1, Tpk2 and Tpk3, they phosphorylated only eight substrates in common and 39 substrates were recognized by any two of the three kinases.

Inspection of the substrate list revealed that known *in vivo* substrates of particular kinases were identified, indicating that this approach is able to successfully identify *bona fide* phosphorylation events. To determine whether novel kinase-substrate interactions on the protein chip represent *in vivo* phosphorylation events, they tested if phosphorylation of several candidate substrates was dependent upon the identified kinase *in vivo*. Using knockout strains and gel-shift assays, about 20 substrates were validated *in vivo*. Therefore, the proteome microarray approach can be used in confidence to identify specific substrates of protein kinases.

The *M. grisea* mitogen activated kinases (MAPKs), cAMP dependent kinases (PKA) and Ca^{2+}-calmodulin dependent kinases (CMKs) play important roles in development, infection-related morphogenesis, and virulence. By performing kinase assays on protein chips containing all *M. grisea* transcription factors, specific substrates will be identified for each kinase tested. Such kinase-transcription factor phosphorylation relationships will provide unique insights into the molecular signals controlling pathogenicity and significantly contribute to our collective ability to interpret results of ongoing expression profiling and large scale mutagenesis studies.

6 Protein-Protein Interactions to Regulate Transcription Factor Activity

In addition to posttranslational modifications, the activity of transcription factors may be affected by the interaction with other proteins, including both DNA-binding and non-DNA-binding proteins as homo- and/or heterodimers. Heterodimerization of transcription factors greatly expands the target sites of the limited number of transcription factors and elevates the sensitivity to fluctuation in the effector concentration. The protein chip approach is ideal to study these interactions because in a single experiment one can identify almost every potential binding partner of a given probe (Zhu et al. 2001). Another advantage of the protein chip approach is its efficiency and speed. For example, to reveal protein-protein interactions among 500 proteins, 125,000 ($= 500 \times 500/2$) of yeast two-hybrid experiments would be required to cover all the combinations (Zhu and Snyder 2002). However, using protein chips, it will take only a few months to perform 500 binding assays, assuming that all the chips are ready. Further, additional co-factors or different experimental parameters (e.g. pH, salt, and temperature) can be easily integrated because of the flexibility of the *in vitro* system.

The construction of the protein-protein interaction map of all *M. grisea* transcription factors will provide a global view of the transcription circuitry and help us to formulate new hypotheses about how the rice blast fungus' infection related machinery is activated upon contact with a host plant at the molecular level.

7 Chromatin Immunoprecipitation (ChIP-Chip)

Gene expression profiling based on DNA/oligo microarrays has proven to be extremely valuable in elucidating expression profiles of suites of genes and generating lengthy lists of candidates involved in various funcitons or cellular activities (Horak et al. 2002). However, DNA microarray expression profiling experiments do not reveal the specific DNA targets of these pathways following a transcription factor activation. An approach termed ChIP-chip allows for the comprehensive identification of targets of transcription factors. Cells are briefly fixed to crosslink transcription factor proteins to the DNA fragments that they bind *in vivo*. The cells are lysed, the chromatin is sheared, and the transcription factor with its associated DNA is immunoprecipitated. The bound DNA fragments are then recovered and fluorescently labeled and hybridized to a DNA chip harboring all the regulatory (intergenic) regions of the genome investigated. This approach identifies all of the bound targets in a single experiment (Iyer et al. 2001; Lieb et al. 2001). It has been used extensively in recent years. For examples, it was applied to identify the targets of Swi4p and Swi6p, the G1/S regulators of yeast, as well as the targets of Ste12p, which is involved in the mating pathway (Horak et al. 2002). A number of important conclusions can be drawn from these experiments. First, ChIP-chip confirmed specific association of factors with known targets (such as cyclins) as well as identified many novel targets. Second, partial overlap of targets was observed for these factors as anticipated since they regulate slightly different aspects of the cell cycle. Third, only a subset of genes whose expression is known to peak during cell cycle was identified. Other subsets are controlled through transcription factors (including HCM1, PLM2, POG1, TOS4, TOS8, TYE7, YAP5, YHP1 and YOX1) whose promoters are bound by Swi4 and Swi6. Importantly, binding was evident in cells growing asynchronously and little change was observed when attempts were made to synchronize cell division. Thus, many transcription factors appear to interact constitutively with their target promoters. A global approach to map out all the potential binding sites of almost every transcription factor in the yeast genome has been launched (Lee et al. 2002; Harbison et al. 2004). On average, each transcription factor in the yeast genome interacts with 38 target genes (range 0–181). Based on the resultant potential binding sequences, they developed various algorithms to identify network motifs, the simplest units of network architecture, and demonstrate that an automated process can use motifs to assemble a transcriptional regulatory network structure. These results show that it is possible to map the regulatory network in considerable depth in yeast and other organisms.

8 Current State of a Transcription Factor Protein Chip and CHIP-Chip Platform for *M. grisea*

The authors of this review are proceeding with the development of a transcription factor protein chip to determine the panel of factors phosphorylated by kinases known to affect appressoria formation and pathogenicity, including PMK1, MPS1, OSM1, CPKA, CPKA, two predicted CaMKs, and one putative protein kinase C. Of the $\sim$ 573 transcription factors annotated, 462 were cloned, expressed and deposited on glass slides in preparation for phosphorylation studies. Concurrently, ChIP-chip studies are being initiated using transcription factors characterized through mutational analysis as being essential for virulence. The available microarray chip for *M. grisea* (G2519F) through Agilent Technologies only contains predicted ORFs, and as such is not appropriate for these studies. A new chip has been designed specifically for use in ChIP-chip studies. This chip, designed by NimbleGen, contains 60–70mer probes spaced every 50 bp tiled across all non-coding regions of the genome after removing highly repetitive sequences. Taken together, these two technologies are poised to advance our understanding of fungal biology and the molecular underpinnings of virulence for this and related fungal pathogens of plants.

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Cellular and Molecular Analyses of Biotrophic Invasion in Rice Blast Disease

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Abstract To cause rice blast disease, *Magnaporthe oryzae* sequentially invades living plant cells using intracellular invasive hyphae (IH) that grow from cell to cell. However, detailed cellular and molecular mechanisms underlying biotrophic invasion are poorly understood. We used live-cell microscopy and fluorescent molecular probes to visualize biotrophic invasion of rice sheath epidermal cells, and demonstrated that IH are sealed in a plant-derived Extra-Invasive-Hyphal Membrane (EIHM) as they grow in first-invaded rice cells and then spread into neighboring cells. The fungus appears to manipulate plasmodesmata for its cell-to-cell movement, based in part on searching behavior of IH before crossing the plant cell wall and on extreme constriction of IH as they cross. Studies of transformed fungal strains that secrete avirulence effector:green fluorescent protein fusions in rice sheath cells led to discovery of a novel pathogen-induced structure, the Biotrophic Interfacial Complex (BIC), that appears to play a role in effector secretion. Optimization of the sheath assay for molecular analysis allowed us to analyze infected rice tissue in which 20% of the RNA came from IH growing in first-invaded cells. This allowed identification of novel candidate effectors by microarray analysis. Next comes understanding dual roles for blast effectors in promoting disease or, in the case of avirulence effectors recognized by rice resistance gene products, in blocking disease.

Keywords Extra-invasive-hyphal membrane · Plasmodesmata · Biotrophic Interfacial Complex · Effector Proteins

1 Introduction

The rice blast fungus has long been considered a hemibiotroph with an initial biotrophic invasion phase followed by a distinct phase of necrotrophic killing of host cells before invasion. A hallmark of biotrophic invasion strategies for prokaryotic

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and eukaryotic pathogens of plants and animals is that these pathogens secrete effector proteins into the cytoplasm of living host cells to block host defenses and control host cellular processes needed for disease. A subset of pathogen effectors, the avirulence (AVR) effectors, includes the *AVR* gene products that are recognized by host resistance (*R*) gene products, resulting in a hypersensitive response that blocks pathogen growth. As with other biotrophic and hemibiotrophic pathosystems, rice blast effectors have been identified so far by their AVR activity. With the exception of *ACE1* (Böhnert et al., 2004), which encodes an appressorium-specific polyketide synthase/nonribosomal peptide sythetase, the cloned blast *AVR* genes, *AVR-Pita1* (Orbach et al., 2000; Khang et al., 2008), *PWL1* (Kang et al., 1995), *PWL2* (Sweigard et al., 1995) and *AVR-CO39* (Peyyala and Farman, 2006) encode small, IH-specific, secreted effector proteins. The best characterized of these, AVR-Pita1, functioned to confer avirulence after transient expression in rice cells (Jia et al., 2000). The abundance of rice blast *R* genes identified suggests that many blast effectors remain to be identified (Ballini et al., 2008).

Extensive structural and functional analyses have been reported for the specialized appressorium used by the blast fungus for mechanically breaching the outer plant surface (Bourett and Howard, 1990; Howard and Valent, 1996; Veneault-Fourrey et al., 2006; Ebbole, 2007). Excellent cytological and ultrastructural analyses have been reported for the blast fungus interacting with host plant cells after penetration (Bourett and Howard, 1990; Heath et al., 1992; Koga, 2001; Rodrigues et al., 2003; Koga and Nakayachi, 2004), although few genes with roles in this colonization have been identified. Once reaching the cell lumen, appressorial penetration pegs expand into filamentous primary hyphae, and form the conduit to transport appressorial components into the primary hypha. In the compatible interaction, primary hyphae differentiate into thicker, beaded invasive hyphae (IH) that fill first-invaded cells and then grow into neighboring cells (Heath et al., 1990). Difficulties in preserving membranes using chemical fixation techniques and transmission electron microscopy (TEM) have led to contradictory reports on the nature of the IH-host cytoplasm interface, with one report suggesting that blast IH were separated from host cytoplasm by invaginated plasma membrane, and another report suggesting that blast IH breached the plant plasma membrane and grew directly in the host cytoplasm (Koga and Horino, 1984; Heath et al., 1990). Elucidating the precise cellular mechanisms by which biotrophic IH colonize living host cells is necessary for understanding effector delivery into rice cells and effector function in promoting rice blast disease or triggering hypersensitive resistance.

2 Invasive Hyphae Are Sealed in Extra-Invasive-Hyphal Membrane as they Invade Living Rice Cells

Defining the interface between blast IH and the rice cytoplasm is critical. We used live cell imaging in optically-clear, excised rice leaf sheaths (Koga et al., 2004), together with fluorescent reporter proteins (Czymmek et al., 2002) and fluorescent

dyes to study biotrophic invasion in the fully susceptible interaction. To determine if blast IH are separated from the rice cell cytoplasm by host membrane, we observed KV1, a transformed rice pathogen constitutively expressing enhanced yellow fluorescent protein (EYFP), as it grew in epidermal cells (Kankanala et al., 2007). Rice membranes were stained with FM4-64 dye, an endocytotic tracker, which inserts into the plasma membrane, moves by lateral diffusion within this membrane, and is actively internalized by the endocytotic pathway. Primary hyphae that remained alive after formation of IH (at $\sim$ 50% of infection sites) internalized FM4-64, which appeared to co-localize with EYFP in the fungal cytoplasm at this level of resolution. This suggested that primary hyphae invaginated the rice plasma membrane, but remained outside this membrane and accessible to dye uptake. In contrast, bulbous IH were precisely outlined by dye (Fig. 1). FM4-64 did not colocalize with EYFP in the IH cytoplasm and was not observed in septa or vacuolar membranes as would be expected if the dye had inserted in the IH plasma membrane. The IH appeared to be protected from dye uptake. We next performed TEM of infected sheath samples prepared by high-pressure freezing and freeze substitution techniques that provide better membrane preservation. The TEM confirmed the presence of plant membrane, the Extra-Invasive-Hyphal Membrane (EIHM), surrounding IH in rice cells. Apparently, differentiation of the primary hypha to IH included sealing the IH in an EIHM-bound compartment where FM4-64 was not able to reach IH cellular membranes.

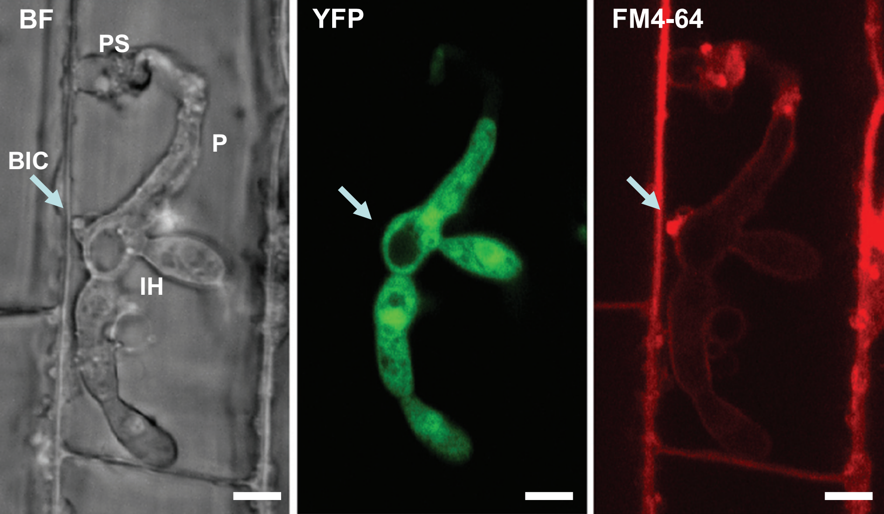

Fig. 1 The EIHM forms a sealed compartment that prevents the endocytotic tracker dye FM4-64 from reaching IH membranes. An IH of EYFP-labeled fungal strain KV1 at 29 hpi is viewed by bright field (BF) optics, and by YFP and FM4-64 fluorescence. At this site, the primary hypha (P) extending from the appressorial penetration site (PS) had lost viability after differentiation into IH. A BIC (*arrow*) beside the first IH cell is rich in FM4-64-stained membranes that are continuous with EIHM. Bar = 5 μm

The EIHM was closely-fitted to the IH cell wall with two prominent exceptions: a dome-shaped “membrane cap” extended ahead of the tips of primary hyphae and filamentous IH before they differentiated into bulbous IH, and EIHM around bulbous IH sometimes enclosed complex aggregations of lamellar membranes and vesicles (Kankanala et al., 2007). Rapid staining of the EIHM by FM4-64 and observed FM4-64-stained vesicles in the vicinity of developing IH have led to the hypothesis that the EIHM is derived from the host plasma membrane through IH-induced endocytotic activity.

Blast IH exhibit pseudohyphal growth for up to 12 h in the first-invaded cells, often filling them up. A time dependent shift in growth pattern occurred at this point and the IH produced tiny penetration-like pegs to cross the plant cell wall. The penetration-like pegs enlarge into filamentous IH, which resemble primary hyphae in size, in filamentous growth style, and in differentiation into bulbous IH. However, unlike primary hyphae, filamentous IH do not internalize FM4-64 and they remain as viable connections between IH in neighboring cells. After the first-invaded cell, IH move through neighboring cells within 2–3 h, rapidly colonizing host tissue.

3 Cell-to-Cell Movement by Biotrophic IH

Live cell imaging confirmed that initial rice cell invasions are biotrophic, because invaded cells appeared healthy and plasmolyzed in sucrose solution, indicating an intact plasma membrane in the cell (Koga et al., 2004). Using the leaf sheath assay, we determined that even third- and fourth-invaded rice cells retain the ability to plasmolyze after IH invasion (Kankanala et al. 2007). At time points up to 49 h post inoculation (hpi), from 75 to 90% of invaded rice cells plasmolyzed. Host cells failed to plasmolyze shortly before IH moved into neighbor cells, indicating that invaded host cells were no longer viable at this time. During plasmolysis, IH were always enclosed inside the shrinking protoplast, consistent with IH being sealed inside an EIHM compartment. These results demonstrated that sequential host cell invasions were biotrophic.

Confocal imaging combined with time lapse experiments demonstrated that IH search along the plant cell wall for specific locations to cross into neighboring cells (Kankanala et al., 2007). The IH appeared to scan along the cell wall until finding an appropriate location to cross. Once found, the IH swells and sends a tiny penetration peg-like structure, an IH peg, across the cell wall. Transmission electron microscopy showed that $\sim$ 90% of locations where the fungus directly contacted the plant wall were pit fields containing clusters of plasmodesmata, and showed examples of the fungus crossing the wall adjacent to normal appearing plasmodesmata. Further analysis showed that IH failed to invade guard cells that lack plasmodesmata. These observations and others strongly suggest that IH exploit functional plasmodesmata in the plant cell wall for movement into living neighbor cells (Kankanala et al., 2007).

4 Secretion of Effectors into Biotrophic Interfacial Complexes

While studying *in planta* secretion of fluorescently-labeled AVR effectors, we discovered a complex, pathogen-induced structure, the Biotrophic Interfacial Complex (BIC), which accumulates these secreted proteins (R. Berruyer, C.H. Khang, P. Kankanala, S.-Y. Park, K. Czymmek, S. Kang and B. Valent, unpublished). The experiments used fungal transformants expressing translational fusions of enhanced green fluorescent protein (EGFP) at the C-terminus of different parts of AVR-Pita1, PWL1 and PWL2 proteins under control of their native promoters. The BIC develops in 2 stages coupled to differentiation of the first filamentous hypha in a host cell into IH (Fig. 2). First, effector:EGFP proteins were secreted into EIHM caps at the

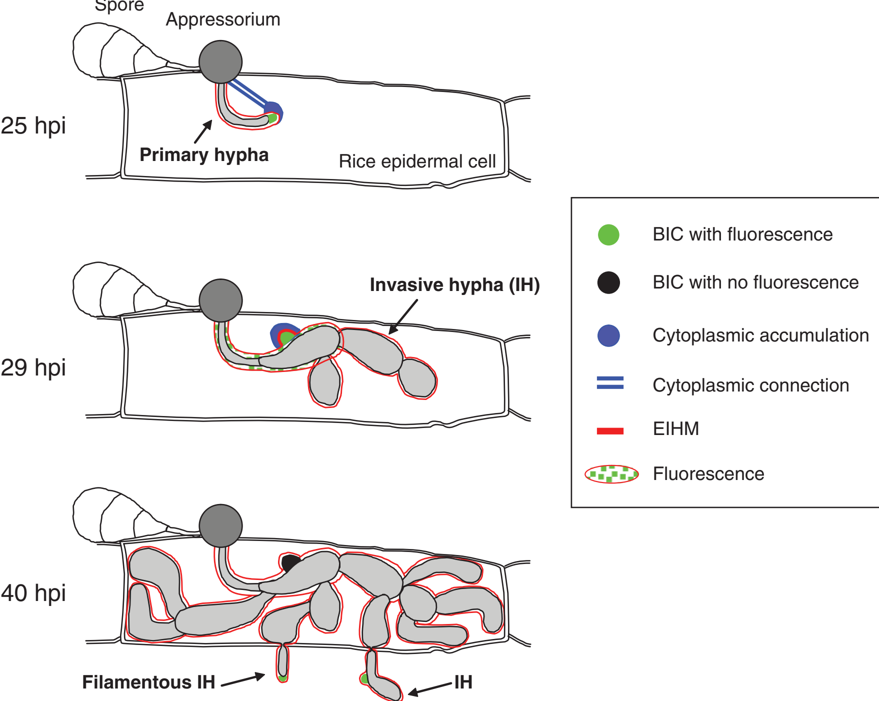

Fig. 2 Summary of the cell biological events involved in biotrophic blast invasion. After appressorial penetration (~ 25 hpi), the fungus grows as a filamentous primary hypha, which invaginates the rice plasma membrane. Effector:EGFP fusion proteins are secreted into the membranous cap BIC at the tips of primary hyphae. By 29 hpi, primary hyphae have differentiated into bulbous IH, which are sealed in an EIHM compartment. The membranous cap BIC moves beside the differentiating IH, and accumulates secreted effector:EGFP proteins as long as IH grow inside the cell. After filling first-invaded cells, IH undergo extreme constriction to cross the plant cell wall, immediately grow as filamentous IH, and then differentiate into bulbous IH (40 hpi). This cycle is repeated for sequentially-invaded rice cells

tips of primary hyphae in first-invaded cells (Fig. 2, 25 hpi) and at the tips of filamentous IH in subsequently-invaded cells (Fig. 2, 40 hpi). When these filamentous hyphae first swelled into IH, the fluorescent membranous caps moved beside the differentiating cell (Fig. 2, 29 hpi) and remained as fixed BIC structures while IH continued to grow in the cell. BIC development was repeated by every hypha that successfully colonized a living host cell. Generally, BIC fluorescence was lost in the invaded cell when the fungus entered a new cell. Effector secretion was targeted to the BIC region while IH continued to grow in a rice cell. That is, effector:EGFP proteins accumulated in BICs and within the EIHM around both hyphal cells involved in BIC development (Fig. 2), and little or no EGFP was visible surrounding IH that subsequently filled the rice cell. Secreted effector fusions partially co-localized with an aggregation of plant endocytotic membranes that labeled with FM4-64 (Fig. 1, arrows). Host cytoplasmic dynamics were focused around both the membranous cap- and IH-BICs during the early hyphal differentiation stage. Correlative light and electron microscopy showed that BICs corresponded to the complex aggregations of lamellar membranes and diverse vesicles previously reported between the IH cell wall and EIHM (Kankanala et al. 2007). Therefore, both known extensions of the blast interfacial matrix corresponded to distinct BIC development stages. These results demonstrate that the first hyphal cells that enter a living host cell have evolved a mechanism to recruit plant cellular components into a distinct interfacial structure. Accumulation of secreted effector:EGFP and dynamic connections with host cytoplasm suggested that BICs are a hub of communication between IH and the host cell, with a likely role in effector secretion into the host cytoplasm.

5 Analysis of the Biotrophic Interaction Transcriptome

Known AVR effector genes encode small, secreted proteins that are specifically expressed during host cell colonization. Therefore, we hypothesized that identification of fungal genes that are specifically expressed by IH during biotrophic invasion would identify additional blast effectors, and that identification of rice genes that are highly up-regulated in the same infected tissues would identify effector-triggered-susceptibility genes. However, identification of fungal and rice genes expressed during biotrophic invasion in leaves has been difficult because so few host cells have encountered the pathogen at early infection stages. Using the excised rice leaf sheath assay, we developed a procedure for obtaining biotrophically-invaded host tissue with 20% IH RNA at 36 hpi when the fungus is predominantly growing in first-invaded cells (Mosquera, 2007). The procedure involved purification of the IH cell type by removal of conidia, appressoria and mycelium from sheath surfaces and removal of hyphae that invaded cut sheath ends. The sheath pieces were trimmed as for microscopy, and then rapidly screened for density of individual infection sites with fluorescent KV1 IH. Densely invaded sheath pieces were frozen for subsequent RNA extraction. Percent of fungal RNAs in these samples was assessed by RT-PCR comparisons of fungal genes in infected tissues versus controls constructed by mixing known proportions of RNA from mycelium grown in nutrient medium and RNA

from mock-inoculated rice. Gene expression in these biotrophically invaded rice tissues was directly compared to control samples with 20% mycelial RNAs and 80% mock-inoculated RNAs, which reflected the ratio of fungal to rice RNAs in infected sheaths, using two-color hybridizations of both whole genome fungal microarrays (#G4137B, Agilent Technologies, Palo Alto, CA) and rice microarrays (#G4138A, Agilent Technologies). Fungal genes with > 50-fold higher expression levels in IH compared to mycelium included *PWL2* and many novel fungal genes encoding small, IH-specific, secreted proteins that are excellent effector candidates. Rice genes that are highly expressed in biotrophically-invaded tissue compared to mock-inoculated tissue are highly enriched in signal transduction components and transcription factors. Rice defense genes were highly up-regulated during blast infection in other studies, but not in our study. Functional analyses of the fungal candidate effector genes and the host effector-triggered-susceptibility genes will begin to associate molecular mechanisms with the cell biology of biotrophic invasion of rice.

6 A New Paradigm in Hemibiotrophy

Our plasmolysis experiments showed that sequential host cell invasions were biotrophic although invaded host cells were no longer alive when the fungus moved into neighbor cells. Complementing these results, Berruyer et al. (2006) followed EYFP-expressing fungal stain KV1 from individual appressoria to development of macroscopically-visible leaf lesions, and reported that fungal growth always preceded visible symptoms. Hyphal swelling and constriction characteristic of biotrophic IH was observed throughout growing lesions. Based on these observations we proposed that rice blast defines a new paradigm in hemibiotrophy in which each successive plant cell invasion is biotrophic, but invaded plant cells die as they fill with hyphae (Kankanala et al. 2007). At least in the highly susceptible interaction, there does not appear to be a distinct switch from biotrophy to necrotrophy as occurs for *Colletotrichum* species (O'Connell and Panstruga, 2006). Necrotrophic hyphae in blast disease probably follow biotrophic invasion in order to utilize remaining nutrients such as host cell walls.

7 Towards Understanding Blast Resistance Mechanisms

The excised rice sheath assay that we have used enhances susceptibility, making it suitable for understanding the fully compatible blast interaction (Koga et al., 2004; Berruyer et al., 2006). Excision of sheath segments has a major impact on resistance responses compared to whole plant infection assays, and compared to a novel intact rice leaf sheath assay (Koga et al., 2004). To assess cellular responses, we compared *Pi-ta*-mediated compatible and incompatible interactions to a non-host interaction between rice and *M. oryzae* strain 4091-5-8, a pathogen of weeping

lovegrass and finger millet, in excised and intact rice leaf sheath assays (Valdovinos-Ponce, 2007). In these studies, the cytological basis for the non-host interaction was dramatically different from *Pi-ta*-mediated resistance. In the excised sheath assay, non-host resistance was characterized by frequent occurrence of crystalline aggregations below appressorial penetration sites, but these aggregations were rare in the intact sheath assay. For the intact sheath assay, non-host resistance was characterized by cytoplasmic fragmentation and/or granulation, which was more representative of responses in whole plant leaf assays. Clearly, excision of sheath segments from the plant altered their cytological responses for incompatible and non-host interactions. Our results support the report of Koga et al. (2004) that intact sheaths are best for studying resistance.

8 Future Directions

We have defined important aspects of the biology of biotrophic blast invasion: (1) by proving that blast IH are sealed inside an EIHM compartment, (2) by demonstrating that blast IH search for locations, apparently plasmodesmata, for crossing into the next living plant cell, (3) by describing the Biotrophic Interfacial Complex, a novel pathogen-induced structure associated with effector secretion in rice blast disease, and (4) by identifying novel IH-specific secreted polypeptides as candidate effectors. It is important to identify the entire set of blast effectors, as well as the subset of AVR effectors that trigger hypersensitive resistance through interaction with rice R proteins. It is important to understand how fungal effectors dampen rice resistance mechanisms, and control rice membrane dynamics and plasmodesmata to execute biotrophic invasion. We must understand how recognition of effectors confers resistance, how effectors are specifically secreted into BICs, and if and how BICs function to deliver effectors to the rice cytoplasm. However, the ultimate challenge lies in translating knowledge of cellular and molecular mechanisms of biotrophic blast invasion into strategies for durable disease resistance.

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Isolation and Functional Analysis of Putative Effectors from *Magnaporthe oryzae* Using Integrated Genomic Approaches

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Abstract Rice blast disease, caused by the fungus *Magnaporthe oryzae*, is a leading constraint to rice production and is a serious threat to food security worldwide. To elucidate the function of effector proteins from *M. oryzae* in pathogenesis and interaction with the host, we have performed RL-SAGE and MPSS approaches to study the gene expression profiles of *M. oryzae* during the interaction. The RL-SAGE and MPSS analyses identified 3,441 and 3,004 annotated *M. oryzae* genes from blast infected rice leaf tissues, respectively. Among them, 217 genes encoding putative secreted proteins, which may play important roles as effectors, were identified. We developed a highly efficient transient protoplast system for gene functional analysis in rice. We present here the detailed procedure of the rice protoplast transient expression system and show the examples of using the system for functional analysis of putative effectors from *M. oryzae*. The combination of RL-SAGE/MPSS genomic profiling approaches with a protoplast functional assay system has provided an efficient approach for large-scale isolation and analysis of effectors from *M. oryzae*.

Keywords RL-SAGE · MPSS · Protoplast system · Secreted protein · *Magnaporthe oryzae*

1 Introduction

Rice blast disease, caused by the fungus *Magnaporthe oryzae*, is a leading constraint to rice production and is a serious threat to food security worldwide. Owing to its economic and social significance, rice blast disease has been extensively studied (Caracuel-Rios and Talbot 2007). *M. oryzae* has emerged as a model system for studying fungus-plant interaction also because of its genetic tractability. The whole genome sequences of both the rice (International Rice Genome Sequencing

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Project 2005) and *M. oryzae* (Dean et al. 2005) are available, providing a wealth of information for functional studies in host-pathogen interaction. *M. oryzae* can reproduce both sexually and asexually, and undergoes a series of developmental steps throughout its life cycle (Dean et al. 2005). Over the past decades, many studies characterized the process of conidia attachment, germ tubes generation, and appresoria formation and penetration (Howard and Valent, 1996; Talbot 2003; Dean et al. 2005). However, there are only limited studies on molecular events that occur after appressoria penetration of the plant cell wall, and the molecular mechanisms underlying the *M. oryzae*-rice interaction remains obscure (Gilbert et al. 2006).

To infect plants, plant pathogenic microbes have evolved mechanisms to enhance microbial fitness by deliver effector molecules into their host plants (Jones and Dangl 2006). These effectors generally contribute to pathogenicity by manipulating biochemical, physiological, and morphological processes in host plants. For instance, pathogen effectors can function in alteration of the host cytoskeleton, signal transduction or suppression of plant defense signaling (Mudgett 2005). Bacterial pathogens use the type III secretion system (TTSS) to deliver effector proteins directly into plant cells (Mudgett 2005). Many studies have focused on determining the functions of a growing number of bacterial effectors. These investigations are leading us to understand how effectors promote bacterial disease by suppressing host defenses (da Cunha et al. 2007). By contrast, effector proteins and the delivery mechanisms in plant pathogenic fungi such as *M. oryzae* are little known (Caracuel-Rios and Talbot 2007). In a recent study, a *MgAPT2* gene was characterized, which encodes a Golgi body-localized component of the polarized exocytosis pathway in *M. oryzae*. Mutants lacking *MgAPT2* were found impaired in the secretion of a range of extracellular proteins. *MgAPT2* is required for fungal pathogenesis and the induction of hypersensitive reaction in an incompatible interaction (Gilbert et al. 2006), demonstrating that the fungal secreted effector proteins play an important role during *M. oryzae*-rice interaction.

Here we present our preliminary data about isolation and functional analysis of putative effectors from *M. oryzae*. We have performed RL-SAGE and MPSS approaches to study the gene expression profiles of *M. oryzae* during both the compatible and incompatible interaction at different time points. The RL-SAGE and MPSS analyses identified 3,441 and 3,004 annotated *M. oryzae* genes, respectively. Among them, 217 in-planta expressed putative secreted protein genes of *M. oryzae* were identified. Moreover, we have established a transient expression system to perform large-scale functional analyses of *M. oryzae* secreted proteins in rice cells based on a recently developed protoplast system (Chen et al. 2006). We describe here the detailed procedure of the rice protoplast transient expression system. We also showed the examples of using rice protoplast transient expression system for functional analysis of putative effectors from *M. oryzae*. The combination of RL-SAGE/MPSS genomic profiling techniques with a protoplast functional assay system, therefore, provides an efficient approach for large-scale isolation and analysis of effectors from *M. oryzae*.

2 Identification of *M. oryzae* Secreted Protein Genes Expressing During *M. oryzae*-Rice Interaction Using RL-SAGE and Massively Parallel Signiture Sequencing (MPSS)

2.1 Deep Transcriptome Analysis of M. oryzae

With the completion of a genome draft sequence (Dean et al. 2005), about 12,841 genes (annotation release 5, http://broad.mit.edu/annotation/fungi/*Magnaporthe*) have been predicted in the genome of *M. oryzae*. However, most of these genes have no experimental support. To validate the annotated putative transcriptional units, we have performed in-depth transcriptome analysis of *M. oryzae* using two widely used quantitative gene expression analysis techniques, RL-SAGE (robust-long serial analysis of gene expression) (Gowda et al. 2004) and MPSS (massively parallel signature sequencing) (Brenner et al. 2000).

SAGE technique is based on the principle that a short-tag corresponds to a unique transcript and the tag frequency reflects the abundance of the mRNA sample. Since its introduction in 1995 (Velculescu et al. 1995), SAGE has pioneered the use of short-tag sequencing methods for genome-wide expression profiling (Vega-Sanchez et al. 2007). Over the past decade, many modifications have been introduced to improve the original technique. While conventional SAGE generates 14 bp tags, many improved techniques, such as LongSAGE (Saha et al. 2002), SuperSAGE (Matsumura et al. 2003), produce longer 21–27 bp tags. In our lab, we have developed an improved method called RL-SAGE which improved the efficiency of LongSAGE library construction by reducing the amount of starting mRNA and the number of ditag PCRs, and increasing the cloning efficiency of concatemer (Gowda et al. 2004). RL-SAGE has been successfully applied to the analysis of rice and maize transcriptomes (Gowda et al. 2007a,b).

MPSS is another tag-based technique for deep transcriptome analysis. MPSS technique is based on the procedure that cDNAs were cloned on microbeads, digested by the enzyme *Dpn* III, and subsequently sequenced to produce 17–20 bp tag signatures by several rounds of hybridizations using adapters with four bases (Brenner et al. 2000). The main advantage of MPSS over other standard tag-based techniques is that it is highly parallel. By using a Solexa sequencing technology (www.Illumina.com), MPSS allows detection of over one million signatures from one experiment (Reinartz et al. 2002). This technology has been applied successfully for gene expression profiling in human (Jongeneel et al. 2003; 2005; Chen et al. 2005), animals (Kim et al. 2007) and plants (Meyers et al. 2004; Nobuta et al. 2007).

The *M. oryzae* strain 70–15 was chosen for transcriptomes analysis since its whole genome sequence is available. Tissues of 70–15 from two different important stages, mycelia and appressoria, were collected for libraries construction and RL-SAGE/MPSS analyses. From mycelia libraries, about 16,580 and 12,531 distinct significant tags were identified by RL-SAGE and MPSS analyses, respectively, while about 12,927 distinct significant tags were identified from appressorium tissue

by MPSS analyses. When matching the identified significant tags to the annotated CDS, 500 bp upstream and 500 bp downstream of CDS, 6,028 and 6,735 unique genes were identified from mycelia RL-SAGE library and MPSS library, respectively, and 7,686 unique genes were identified from appressorium MPSS library. Taken together, RL-SAGE and MPSS analyses identified more than 9,000 genes, representing over 80% of the predicted genes in *M. oryzae* genome. The comparative analysis between mycelia MPSS library and appressorium MPSS library also identified 7,135 mycelium-specific and 7,531 appressorium-specific significant tags, corresponding to 2,088 and 1,784 unique genes, respectively (Gowda et al. 2006). These results, therefore, provided a useful genomic resource for future functional characterization of *M. oryzae* genes involved in fungal growth, development and fungus-plant interactions.

2.2 In-Planta Expressed Secreted Proteins of M. oryzae

To identify *M. oryzae* genes that are involved in fungus-plant interactions, we constructed one RL-SAGE library with RNA isolated from the rice leaf tissues after 96-h compatible blast infection. For MPSS analysis, we constructed 11 libraries from both incompatible and compatible interactions. The RL-SAGE and MPSS analyses identified 3,441 and 3,004 annotated *M. oryzae* genes that were expressed in blast infected leaves, respectively. In total, 4,923 annotated *M. oryzae* genes were identified by two approaches together.

Previous studies have shown fungal secreted proteins play crucial roles during fungi-plants interaction (Rep 2005; Ellis et al. 2007). These secreted proteins are broadly referred to as "effectors". These effectors may alter host cell structure or function and influence pathogenicity or defense response. The whole genome sequencing identified 739 proteins that are predicted to be secreted by *M. oryzae* (Dean et al. 2005). After the RL-SAGE and MPSS analyses, we then focused on the identification of *M. oryzae* secreted protein genes. From 4,923 annotated genes identified by RL-SAGE and MPSS, 217 genes were found encoding predicted secreted proteins. These putative secreted proteins, therefore, may play important roles during *M. oryzae*-rice interaction.

3 Using Rice Protoplast Transient Expression System for Functional Analysis of Putative Effectors from *M. oryzae*

3.1 A Highly Efficient Transient Protoplast System for Gene Functional Analyses in Rice

The growing genomic data have propelled gene functional characterizations in plants, yet the majority of the genes have not been experimentally identified. Basically, the functional analysis of genes requires ectopic expression of candidate genes

in plant cells to provide a phenotype that helps to elucidate its function. The generation of stable transgenic lines has offered a powerful tool for investigating gene functions in plants. However, the relatively expensive and time-consuming process for stable transformation limits the utilization of this approach for large-scale assays. By contrast, the use of transient expression assays offers an opportunity to study a large numbers of genes in plants quickly. Recently, we have developed a significantly improved rice protoplast transient expression system for gene functional analyses (Chen et al. 2006).

Plant protoplasts are usually isolated from leaf tissue or suspension culture cells. Leaf tissue-derived mesophyll protoplasts retaining their cell identity and differentiated state have been proven to be a versatile system for studying gene function in several plant species, such as maize, Arabidopsis (Sheen 2001), and tobacco (Tao et al. 2002). In rice, however, the isolation of mesophyll protoplasts from rice is difficult due to its leaf structure. For a long time, rice protoplast system is only available for some varieties based on suspension cultures. However, the isolation of large-scale protoplasts is difficult for many rice varieties due to the difficulties in induction of the suspension culture cells, and the protoplasts isolated from suspension culture cells might not be suitable for special signal transduction pathway assays due to absence of some essential components in the process. With the aim to establish an efficient protocol for rice protoplasts isolation, we tested different tissues as sources, and found that a large amount of rice protoplasts could be isolated from stem and sheath tissues but not from leaves (Fig. 1). The detailed procedure for the highly efficient protoplast-based transient system for gene functional expression studies in rice is described below (Chen et al. 2006).

3.2 Detailed Procedure for Rice Protoplast Transient Expression System

Based on our protocol, more than 10×10^6 protoplast cells could be easily obtained from about 100 two-week-old rice seedlings. It is sufficient for more than 50 common transfection experiments (2×10^5 cells can be used per transfection). Our results also showed that the protoplast system is ideal for RNA and protein assays, such as RT-PCR and western blotting (Chen et al. 2006).

3.2.1 Plant Materials

About 100 dehusked rice seeds are sterilized by using 40% Clorox for 20 min. Rice seeds are germinated on 1/2 MS medium after washing with sterile H_2O 10 times. Rice seedlings are grown at 26 °C in the dark for about 2 weeks. Etiolated rice tissues are easier for protoplasts manipulation. However, the green rice tissues can also be used for protoplast manipulation if necessary, although the protoplasts from green tissues might be more fragile. We have tested many different varieties such as japonica TP309, Nipponbare, and indica IR64 for protoplast isolation, and found they are all suitable for protoplast isolation.

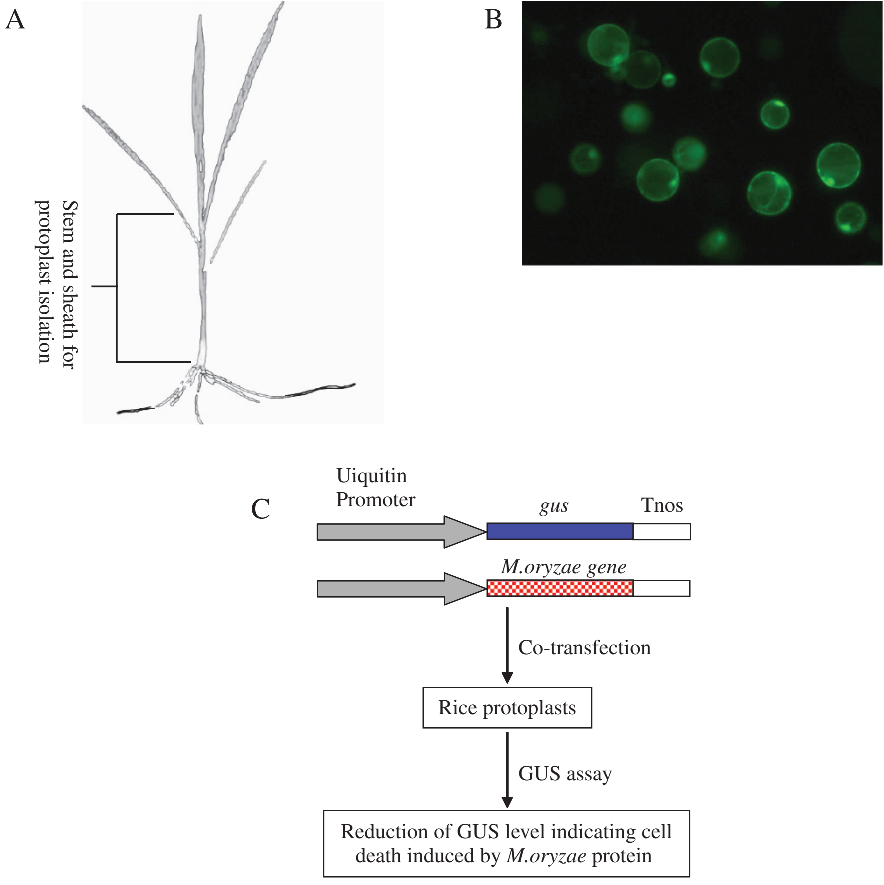

Fig. 1 Using rice protoplast transient expression system for functional analysis of putative effectors from *Magnaporthe oryzae*. (**A**) Stem and sheath tissues of young rice seedlings suitable for isolation of large amount of protoplasts. (**B**) High efficiency of gene tranfection and expression in rice protoplasts. Rice protoplast tranfection efficiency was checked using a GFP driven by the constitutive 35S promoter. The percentage of the transfected cells is routinely scored in the range between 50% and 70%. (**C**) A flow chart of using protoplast transient expression system for detecting rice cell death induced by *M. oryzae* secreted proteins

3.2.2 Protoplast Isolation

Using a razor blade, the stems including the sheaths of the seedlings are cut into $1 \sim 2$ mm pieces inside the hood. The chopped tissues are put into a petri dish with 12 ml Enzyme Solution (Table 1) till they cover the surface of medium. Usually 3 petri dishes of chopped tissues can be made from 100 seedlings.

Apply vacuum to the tissues for 1 h at 20 mm Hg for infiltration of the Enzyme Solution (Table 1), followed by incubation in the dark with gentle shaking ($\sim$ 40 rpm) at room temperature for about 4 h to digest the tissues. The digestion

Table 1 Media and solutions used for rice protoplast system

Enzyme Solution	1.5% cellulase, 0.3% macerozyme in K3 Medium. Filter sterilized, store at −20 °C		
K3 Medium	10 × B5 Macro 100 ml, 100 × B5 Vitamines 10 ml,	100 × B5 Micro (I) 10 ml, 200 × MES (0.1g/ml) 5 ml,	1000 × B5 Micro (II) 1ml, 500 × myo-inositol (0.05 g/ml) 2 ml,
	100 × NH_3NO_3 (25 mg/ml) 10 ml,	100 × $CaCl_2$ (75 mg/ml) 10 ml,	100 × Xylose (25 mg/ml) 10 ml,
	0.4 M Sucrose 137 g.	Adjust pH 5.6–5.8 by 1 M KOH, filter-sterilized, store at 4 °C.	
W5 Medium	154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES. Adjust pH 5.7 by 1 M KOH, filter, store at 4 °C.		
Suspension Medium	0.4 M Mannitol, 20 mM $CaCl_2$, 5 mM MES. Adjust pH 5.7 by 1 M KOH, filter, store at 4 °C.		
PEG Solution	40% PEG 4000, 0.4 M Mannitol, 100 mM $Ca(NO_3)_2$. Adjust pH7.0 by 1 M KOH, filter, store at −20 °C.		
10 × B5 Macro (1 L)	KNO_3 25 g, $(NH_4)_2SO_4$ 1.34g, $CaCl_2 \cdot 2H_2O$ 1.5 g, $MgSO_4 \cdot 7H_2O$ 2.5 g, $NaH_2PO_4 \cdot H_2O$, 1.5 g.		
	Dissolve $CaCl_2$ and other ingredients in separate beakers, then combine and bring to 1 Liter, store at 4 °C.		
100 × B5 Micro (I) (1 L)	Dissolve well of $MnSO_4 \cdot H_2O$ 0.78 g and $ZnSO_4 \cdot 7H_2O$ 0.2 g in 400 ml ddH_2O; Dissolve H_3BO_3 0.3 g and KI 0.075 g in 400 ml ddH_2O. Slowly combine two solutions, add ddH_2O to a final volume 1 L, store at 4 °C.		
1000 × B5 Micro(II)(1 L)	Dissolve $NaMoO_4 \cdot 2H_2O$ 0.250 g, $CuSO_4 \cdot 5H_2O$ 25 mg, $CoCl_2 \cdot 6H_2O$ 25 mg in ddH_2O to a final volume 1 L, store at 4 °C.		
100 × B5 Vitamins (1 L)	Dissolve Vitamin B1 (Thiamine-HCL) 1g, Vitamin B6 (Pyridoxine-HCl) 0.1 g, and Nicotinic Acid 0.1 g in ddH_2O to a final volume 1 L, store at 4 °C.		

duration is optimized according to our experiments. We have tested different time points for tissue digestion effects. The protoplast cells obtained increased significantly when longer digestion duration is used, but finally reaches a stable yield level after 4 h digestion (Chen et al. 2006).

The Enzyme Solution is gently removed from the tissues using a glass pipet, then add 10 ml W5 Medium to each petri dish. The Enzyme Solution contains high concentration of sucrose, and it may cause the protoplasts to float at the top of medium. The replacement of the Enzyme Solution with W5 Medium (Table 1) therefore allows the protoplasts to be centrifuged at the bottom of the tubes for recovery.

The protoplasts are released by shaking at 80 rpm for 1 h. The W5 medium containing protoplasts are filtered with a 35 μm nylon mesh. The filtered medium are transferred into round-bottomed 8 ml glass tubes. The protoplasts are collected by spinning at 150 × g for 4 min at room temperature. Higher centrifuge speed may be used if the protoplast collection is poor. However, the protoplasts may

be damaged if the speed is higher than 300 × g. The protoplasts can be used for transfection after removing W5 medium and re-suspending in Suspension Medium (Table 1).

3.2.3 Protoplast Transfection

Plasmid DNA is prepared in 40% PEG solution before the protoplasts are ready for transfection. 40% PEG solution is usually stored at −20 °C. The PEG solution is thawed in a hot water bath at about 70–80 °C, and let it cool down at room temperature for use.

About 10 μg DNA is prepared in a 2.0 ml tube for each construct. Then 200 μl (usually $1.5 \sim 2.5 \times 10^6$ cells/ml) of suspended protoplasts are added to the tube. Add 220 μl 40% PEG solution and mix it well immediately by gently shaking, then incubate 20 min at room temperature.

To dilute PEG, 1.0 ml K3 medium is added to the tube to check fluorescence microscopy of the transfected protoplasts. The K3 medium added to the transfected protoplasts samples can increase the solution density and float the protoplasts from the sedimented dead ones. Again 1.0 ml W5 medium is added to dilute PEG for other assays of protoplasts, such as Luciferase activity, GUS detection. The transfected samples are incubated for over night (12 h or longer) for microscopy observation or other assays.

3.2.4 Transfected Protoplast Detection

Fluorescence Microscopy: The protoplasts from the top layer of the solution are taken for the fluorescence observation using a Nikon E600 fluorescence microscope. GFP fluorescence is visualized with a filter set consisting of an excitation filter of 450–490 nm, a dichroic mirror of 510 nm, and a barrier filter of 520–560 nm. DsRed fluorescence is visualized with a filter set consisting of an excitation filter of 540–580 nm, a dichroic mirror of 595 nm, and a barrier filter of 600–660 nm. Images are captured with a SPOT 2 Slider charge-coupled device camera and the associated software (Diagnostics Instruments, Sterling Heights, MI).

Luciferase/GUS activity Assays: The transfected protoplasts are collected by centrifuging at 5,000 rpm for 5 min. The supernatant is removed carefully. Add 200 μl 1 × CCLR (Cell Culture Lysis Reagent from Luciferase Assay System) (Promega, Madison, WI, USA) to the tubes. The protoplasts are vortexed vigorously for 30 s, then centrifuge at 5,000 rpm for 5 min. Take the supernatant cell lysate for both LUC and GUS assay.

To perform Luciferase activity assay, 40 μl cell lysate is transferred to the plate and 100 μl of the Luciferase Assay Reagent (Promega, Madison, WI, USA) are added to the cell lysate. Then the LUC activity is tested with a luminometer.

To perform GUS activity assay, 100 μl cell lysate is transferred to the plate and add 100 μl of 2 × GUS assay buffer (Jefferson et al. 1987) and mix well. The assay mix is incubated at 37 °C in the dark for about 2–4 h. The reaction is stopped by

adding 0.2 M. Na_2CO_3 to 20 μl of assay mix. Then detect the assay mix with a fluorometer.

3.2.5 Using the Rice Protoplast Transient Expression System for Functional Analysis of Putative Effectors from *M. oryzae*

Transient gene expression systems have provided a rapid approach to investigate elicitor activity of pathogen effectors in plants (Mindrinos et al. 1994; Jia et al. 2000). By co-introducing a GUS gene as a reporter marker, and detecting the reduced GUS expression as an indicator of cell death, Jia et al. (2000) analyzed the function of AVR-Pita in rice cells using particle bombardment-mediated transient expression assay. We modified the transient expression system to analyze the function of the *M. oryzae* secreted proteins in rice cells based on the protoplasts system. The use of protoplast system has several advantages over the use of bombardment-mediated system, including high efficiency and repeatability of transfection, and easy assay of transfected samples. To express *M. oryzae* secreted protein genes in rice protoplasts for functional analysis, a plant expression vector has been constructed utilizing a maize ubiquitin promoter (Christensen and Quail 1996) to drive high-level expression of interest genes. Some of the in-planta expressed *M. oryzae* secreted protein genes were selected to clone into the vector, and the constructs were co-tranfected into rice protoplasts with another plasmid expressing a GUS reporter gene. GUS activity was assayed after overnight incubation. Reduced GUS level of the protoplast samples would be observed if expression of the *M. oryzae* secreted protein caused rice cell death (Fig. 1C). By using this system, we are able to perform large-scale functional analysis of *M. oryzae* secreted protein in rice cells, and several rice cell-death inducing proteins have been identified (Unpublished data).

4 Conclusions and Future Perspectives

The RL-SAGE and MPSS analyses identified 4,923 annotated *M. oryzae* genes from rice blast infected leaf tissues. Among them, 217 genes encoding putative secreted protein were identified. These candidate effector proteins may play important roles in the *M. oryzae*-rice interaction. By using the GUS activity level as a cell death indicator in rice protoplasts, we established a rice protoplast transient expression system to identify the secreted proteins with elicitor activity. Through these integrated approaches, several cell-death inducing *M. oryzae* effector proteins have been identified. Our results indicate that combining RL-SAGE/MPSS genomic profiling with protoplast functional assays can facilitate the identification of effectors from *M. oryzae*. Further identification of more effectors and functional characterization of those proteins and their host targets will provide new insights into the function of these proteins in the interaction between rice and *M. oryzae*.

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Searching for Effectors of *Magnaporthe oryzae*: A Multi-Faceted Genomics Approach

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Abstract In 2005, a draft sequence of *Magnaporthe oryzae* isolate 70–15 genome was published (Dean et al. 2005). Complete rice genome sequence was also published in the same year (International Rice Genome Sequencing Project, 2005). As a result, study of the *Magnaporthe*-rice interaction has entered the "post-genomics" era. The challenge now is how to make use of this large amount of information to improve our understanding of this interaction. By employing genomics information, we are trying to identify and characterize *Magnaporthe* effectors directly involved in molecular interactions between the pathogen and the host. In this paper, we present a short review of phytopathogen effectors and *Magnaporthe* avirulence factors. We then briefly describe our current approach to identify new effectors from *Magnaporthe*.

Keywords DNA polymorphisms · Effector · Signal peptide · SuperSAGE

1 Pathogen Effectors

It is now widely recognized that plant pathogens utilize a battery of proteins that function to compromise host defense mechanisms, thereby increasing the pathogens' fitness. Such pathogen proteins are called "effectors" (see Kamoun 2007 for a review). The concept of "effector" covers those of "virulence" and "avirulence" factors in the traditional definition. Most "avirulence" factors can be perceived as a subset of effectors that are recognized by the hosts, thereby triggering the defense response. Recent studies of pathogen effectors are providing exciting new information that is important for understanding not only the mechanisms of pathogenesis, but also the defense signaling pathways in plants. Viruses encode various proteins that function to suppress gene-silencing mechanisms of plants (Voinnet 2005). Gram-negative phytopathogenic bacteria deliver a wide array of effectors inside host cells by the type III secretion system (TTSS). Some bacterial effectors

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were shown to function in suppressing host defenses (see da Cunha et al. 2007). Recently, effectors of filamentous pathogens, including those of oomycetes, basidiomycetes and ascomycetes, have been the focus of attention (see Kamoun 2007; Ellis et al. 2007).

2 Fungal Effectors

In this section, we provide a brief update on fungal effectors. So far, the most intensively studied species is the basidiomycete fungus flax rust, *Melampsora lini* (Ellis et al. 2007). From haustorially-expressed genes coding for secreted proteins, four avirulence factors, *AvrL567*, *AvrM*, *AvrP123* and *AvrP4*, that confer the HR in flax cultivars that harboring cognate R-genes were isolated (Dodds et al. 2004; Catanzariti et al. 2006). These avirulence proteins have no known conserved domains, and the corresponding genes are highly polymorphic, suggesting that they might have undergone diversifying selection. *Ustilago maydis* is a pathogenic basidiomycete fungus causing the smut disease in maize. The sequenced *U. maydis* genome contained 12 clusters of genes encoding small secreted proteins with unknown function (Kämper et al. 2006). These genes are specifically induced in host tumor tissues. Deletion of individual clusters resulted in changes in virulence, suggesting that a battery of small secreted proteins are involved in manipulation of host cells to establish biotrophy. Kemen et al. (2005) showed that the rust *Uromyces fabae* (basidiomycete) expresses a gene for secreted protein Uf-RTP1p in haustoria. By immunocytological study, they showed that Uf-RTP1p is localized in host plant nuclei, being the first direct demonstration of translocation of a fungal effector into host cells. Two avirulence genes, Avr_{a10} and Avr_{k1}, of the powdery mildew fungus, *Bulmeria graminis* f sp *hordei* (ascomycete), were identified that confer resistance to barley with cognate *R*-genes (Ridout et al. 2006). A transient expression study showed that these factors function inside host cells. These avirulence proteins are interesting in two aspects: (1) they do not have secretion signals, so they must be secreted by the pathogen via a yet unidentified mechanism, and (2) their overexpression in host cells confers enhanced pathogen virulence, suggesting that these avirulence factors are indeed virulence effectors in the absence of cognate *R*-gene. *Cladosporium fulvum*, an imperfect fungus, is a pathogen of tomato. An avirulence determinant *Avr2* of *C. fulvum* is a secreted cysteine-rice protein recognized by the tomato *R*-gene *Cf-2* (Dixon et al. 1996; Luderer et al. 2002). Avr2 was shown to bind and inhibit tomato secreted cysteine protease Rcr3 (Rooney et al. 2005). Therefore, it is hypothesized that Avr2 functions as a virulence factor in the absence of *Cf-2*.

3 Host Species Specificity Determinants and Avirulence Genes of *Magnaporthe*

Here we provide a brief overview of the current knowledge on *Magnaporhte* factors that determine host species specificity and avirulence to host cultivars, since they comprise a subset of *Magnaporthe* effectors.

Most *M. oryzae* rice pathogens cannot infect weeping lovegrass (*Eragrostis curvula*). By map-based cloning, Sweigard et al. (1995) identified *PWL2*, a gene responsible for the non-pathogenicity of rice pathogens against weeping lovegrass. *PWL2* encoded a glycine-rich, hydrophilic protein with a putative secretion signal sequence. The virulence function of PWL2 is not known. The same authors isolated *PWL1* that determines the same host specificity as *PWL2* (Kang et al. 1995). *Avr-Pita*, isolated by map-based cloning, was shown to trigger the HR in rice cultivars harboring the *R*-gene *Pi-ta* (Orbach et al. 2000). *Avr-Pita* encoded a metalloprotease with a secretion signal and proprotein sequences at its N-terminus. When transiently expressed inside rice cells, only processed mature protein AVR-Pita$_{176}$ without the N-terminal sequences was shown to trigger the HR, suggesting that AVR-Pita is recognized inside plant cells. AVR-Pita$_{176}$ was shown to directly interact with rice Pi-ta by yeast 2-hybrid and in vitro binding assays corroborating the idea of intra-cellular interaction between Avr-Pita and Pi-ta (Jia et al. 2000). So far, no virulence function of Avr-Pita has been reported.

The *Avr1-CO39* gene was derived from a *M. oryzae* isolate from weeping lovegrass and it controls avirulence on rice cultivar CO39 (Farman and Leong 1998; Farman et al. 2002; Tosa et al. 2005). *Avr1-CO39* avirulence function was localized to a 1.05-Kb region of DNA, but the corresponding protein has not yet been clarified.

ACE1 is an avirulence factor recognized by the rice *R*-gene *Pi33* (Bohnert et al. 2004). *ACE1* encodes a polyketide synthase fused to a nonribosomal peptide synthetase. *ACE1* is remarkable in that its metabolite, not the ACE1 protein itself, must be the avirulence determinant recognized by the host plant.

4 Towards the Isolation of *Magnaporthe* Effectors

The above review of pathogen effectors highlights several points that help us identify more *Magnaporthe* effectors: (1) the majority of effectors of filamentous pathogens are secreted proteins, although there are notable exceptions; (2) effectors tend to be specifically expressed during infection, and (3) effector protein genes appear to be under diversifying selection. We will use these criteria to identify novel effectors from *Magnaporthe*.

4.1 Bioinformatics Identifies Putative Secreted Proteins

It is logical to assume that effector proteins must be secreted from pathogens before exerting their function in host plants. Bioinformatics tools are available to predict secreted proteins from their amino acid sequences. The most widely used program is SignalP (Nielsen et al. 1997). SignalP was successfully used to predict secreted proteins in *Phytophthora* (Torto et al. 2003). We applied SignalP to 12,840 predicted amino acid sequences of *M. oryzae* proteins deposited in GenBank, and identified $\sim$1, 884 putative secreted proteins.

4.2 *SuperSAGE Reveals* Magnaporthe *Genes Expressed During the Interaction*

We tried to identify *M. oryzae* genes that are transcribed during the infection process. SuperSAGE (Matsumura et al. 2003) is an improved version of Serial Analysis of Gene Expression (SAGE; Velculescu et al. 1995) that allows the isolation of 26-bp tag fragments instead of 15-bp (original SAGE) or 21-bp (LongSAGE; Saha et al. 2002) tag fragments. The number of occurrences of a tag represents the transcript abundance, and tag sequence provides information for a tag-to-gene annotation. Since sequence specificity of 26-bp tag is high enough, we can apply SuperSAGE to biological materials whereby two or more eukaryotic organisms are mixed. After tag isolation, we can assign each tag to the DNA sequences of the involved species. To see the expression of *Magnaporthe* as well as rice genes at the focus of interaction, we are applying laser microdissection (LMD; Emmert-Buck et al. 1996) to isolate single rice cells attacked by *M. oryzae*. So far five SuperSAGE libraries were made from *M. oryzae*-infected rice for compatible and incompatible interactions at different times after inoculation. Putting information from all libraries together, we found $\sim 5,000$ *M. oryzae* genes are expressed in the infection focus.

Combining the information of putative secreted protein genes and that from SuperSAGE expression, we identified about 450 *M. oryzae* genes that code for putatively secreted proteins and are expressed at the focus of interaction. We set out to study the function of these genes.

4.3 *DNA Polymorphism Study of* M. oryzae

The level of DNA polymorphism within a species is determined by mutation rate, effective population size and natural selection imposed on the DNA region. If purifying selection (negative selection) is acting on the region, the amount of DNA polymorphism tends to be low. In contrast, if the region is under diversifying selection (one of positive selection), the DNA polymorphism level tends to be inflated. Looking at gene coding regions, we can divide base substitutions into two categories: non-synonymous and synonymous ones. The numbers of non-synonymous changes (Ka) and synonymous changes (Ks) gives a hint about the natural selection imposed on the regions. The ratio $\mathrm{Ka/Ks} < 1$ indicates the locus is under purifying selection, $\mathrm{Ka/Ks} > 1$ suggests positive selection. We can use such criteria to find the loci that are presumably involved in biotic interactions.

To facilitate mutation detection, we are using EcoTILLING (Till et al. 2003; Comai et al. 2004). EcoTILLING using bulked DNA allows us to detect mutations in a cost-effective manner. Preliminary data tells us that the DNA polymorphism level of *M. oryzae* is extremely low ($\phi < 10^{-5}$). This may suggest a small effective size of the species reflecting the recent expansion of *M. oryzae* population over the world. Through this survey, we found that *Avr-Pita* (Orbach et al. 2000) is one of the

most polymorphic loci among the all studied loci. Moreover, we identified several loci exhibiting Ka/Ks > 1. We are currently studying their functions in detail.

4.4 Functional Tests of Putative Effector Genes

To study the function of candidate *M. oryzae* effector genes, we are employing (1) knockout experiments of putative effector genes in *M. oryzae* by homologous recombination, (2) transient overexpression of the *M. oryzae* genes in *Nicotiana benthamiana*, (3) stable overexpression of the *M. oryzae* genes in rice.

5 Perspectives

We anticipate an exciting decade ahead whereby understanding the function of many *Magnaporthe* effectors will uncover hitherto unknown mechanisms of pathogen infection, which in turn will lead to the identification of novel host defense mechanisms. Now the post-genomics era of studies of *Magnaporthe*-rice interactions has really started.

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Developing Resources for Analysis of Secreted Proteins from *Magnaporthe oryzae*

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Abstract Extracellular proteins of fungal pathogens are candidate effector molecules. We have cloned a large number (∼300) of putative secreted proteins from *Magnaporthe oryzae*. The proteins were fused to a His(×6) tag to allow for affinity purification of the proteins. The genes were transformed into *M. oryzae* to test for expression. Approximately one-third of the genes could be expressed and secreted to sufficient levels to allow detection by western blot analysis of culture filtrates. In most cases, constitutive expression of the proteins in the transformed strains did not markedly alter their interaction with rice. However, a number of transformed lines may have reduced aggressiveness as a result of protein expression. Proteins were purified from culture filtrates of *M. oryzae* and symptoms were observed on rice leaves in only very few cases. The results to date are summarized and the resources developed in the project are being used for functional analysis of secreted protein. The resources generated are being distributed to facilitate functional characterization of the secretome of *M. oryzae*.

Keywords Extracellular · Secreted · Protein expression · Pathogenicity · Genomics

1 Introduction

We are developing tools to examine the interaction between *Magnaporthe oryzae* and its host plant. Secreted proteins represent candidate molecules for virulence factors as well as factors that may be recognized by the host rice plant to trigger defense responses. For oomycete pathogens of dicots, approaches to analyze virulence factors have been developed using vectors to express proteins in planta for analysis of plant responses. These studies tested specific proteins or screened libraries of cDNAs for proteins involved in plant interactions (Laugé et al., 2000; Takken et al.,

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2000; Kamoun et al., 2003). These approaches are now being employed to examine fungal plant pathogens. Previously, we reported the cloning of nearly 300 genes encoding putatively secreted proteins and transformation of *M. oryzae* with these genes for expression (Lu et al., 2004). We have examined a number of secreted proteins from *M. oryzae* to directly assess their ability to evoke plant responses. In our initial studies, we attempted to screen proteins in an unbiased manner and found few proteins that evoke responses on rice plants. However, mutational analysis of some of the genes encoding the proteins suggest they function in virulence and additional genes are being targeted for analysis. We report a summary of genes cloned and expressed in *M. oryzae* and discuss future efforts to prioritize proteins for analysis.

2 Secreted Protein Gene Expression

We previously reported on the cloning, expression and methods for characterization of proteins (Lu et al., 2004). A summary of cloned genes and transformed *M. oryzae* isolates is shown in Table 1. Plasmids for expression of proteins with the *M. oryzae* ribosomal protein 27 promoter were produced in a vector containing the hygromycin B resistance marker. These were transformed into *M. oryzae* strain 70–15 and these transformants are available from Texas A&M University (DE). The *M. oryzae* genes were amplified from genomic DNA and contain introns in most cases. Plasmids for expression of the *M. oryzae* cDNAs for some of these genes in *Pichia pastoris*, and transformed *P. pastoris* containing these plasmids, are also available.

To functionally test secretion of proteins, the candidate genes were expressed in *M. oryzae* with an $RGSH_6$ (Arg Gly Ser His $\times$ 6) tag for western blot detection and protein purification. Each PCR product was cloned into pDL1 by recombination cloning in yeast to produce the fusion with the 6 $\times$ His tag at the C-terminus. DNA isolated from yeast cells was used to transform *E. coli* cells. We routinely characterize three colonies to screen for the construct. Approximately 75% of *E. coli* colonies contained the correct clone. This high efficiency of cloning is necessary for high-throughput analysis of our 300 target genes.

We also expressed several genes in *P. pastoris* using the Invitrogen *P. pastoris* expression system (Cat. #K1710-01). Clones in pDL1 lacking introns were amplified using universal primers for pDL1 (and containing the 6 $\times$ His tag) modified with restriction sites to allow cloning into pIC3.5 for expression in *P. pastoris*.

3 Secreted Protein Gene Analysis

Using SignalP (Nielsen et al., 1997; Nielsen & Krogh, 1998), we identified a group of 1238 potentially secreted proteins from the first release of the *M. oryzae* genome. ProtSort (Softberry.com) was used as a second method that further identified 758 of the 1238 candidates as being secreted. Criteria such as predicted size, representation

Table 1 Summary of Magnaporthe oryzae genes cloned

GENE ID	Sequence similarity	E value	Plasmid	Path	Prot	EST
MGG_00043	DUF995 (PF06191.3)	2.E-01	pGLH241	NT	N	N
MGG_00066[1]	DUF1996 (PF09362.1)	<E-99	pGLH37	NT	N	N
MGG_00081	conserved hypothetical (N. crassa)	8.E-28	pGLH36	9	Y	Y
MGG_00194	conserved hypothetical (N. crassa)	<E-99	pGLH79	5,7	N	N
MGG_00210	conserved hypothetical (N. crassa)	2.E-79	pGLH78	NA	NA	Y
MGG_00216[1]	unique		pGLH203	3,5	N	N
MGG_00263	endo-1,3-beta-glucosidase	5.E-64	pGLH77	NT	NT	Y
MGG_00269	unique		pGLH242	NA	NA	N
MGG_00311	Peptidase_A4 (PF01828.8)	8.E-10	pGLH75	5,7	Y	N
MGG_00314	COesterase (PF00135.19)	2.E-81	pGLH74	NT	N	H
MGG_00486[1]	WSC (PF01822.10)	3.E-21	pGLH181	NA	NA	N
MGG_00495	conserved hypothetical (N. crassa)	1.E-24	pGLH69	7,9	N	Y
MGG_00505	SUN (PF03856.4)	7.E-52	pGLH125	NT	N	Y
MGG_00527	conserved hypothetical (N. crassa)	2.E-09	pGLH124	3,9	Y	Y
MGG_00570	conserved hypothetical (N. crassa)	<E-99	pGLH128	NT	NT	N
MGG_00614	unique		pGLH244	NA	NA	N
MGG_00618[1]	Pectinesterase (PF01095.10)	1.E-22	pGLH66	7,9	Y	Y
MGG_00732	DUF295 (PF03478.9)	5.E-01	pGLH217	5,7,9	Y	N
MGG_00737	conserved hypothetical (N. crassa)	2.E-12	pGLH58	NA	NA	Y
MGG_00756	conserved hypothetical (N. crassa)	1.E-31	pGLH56	NA	NA	Y
MGG_00779	GMC_oxred_N (PF00732.10)	2.E-23	pGLH113	7,9	NT	Y
MGG_00888[1]	conserved hypothetical (N. crassa)	8.E-12	pGLH112	NT	N	N
MGG_00994[1]	Glyco_hydro_47 (PF01532.11)	<E-99	pGLH111	9	Y	N
MGG_01009	conserved hypothetical (N. crassa)	3.E-20	pGLH228	9(+)	N	Y
MGG_01146	Cutinase (PF01083.13)	1.E-89	pGLH19	NA	NA	N
MGG_01173	Hydrophobin_2 (PF06766.2)	2.E-39	pGLH236	9	Y	H
MGG_01219	conserved hypothetical (N. crassa)	4.E-34	pGLH105	3	N	N
MGG_01247[1]	Glyco_hydro_18 (PF00704.19)	1.E-92	pGLH9	9,3	Y	N
MGG_01367[1]	Phospholip_A2_3 (PF09056.2)	3.E-71	pGLH104	9	N	N
MGG_01403	Glyco_hydro_62 (PF03664.4)	3.E-10	pGLH103	7,9	Y	H
MGG_01466[1]	WSC (PF01822.10) (N. crassa)	4.E-18	pGLH8	9	N	Y
MGG_01603	conserved hypothetical (N. crassa)	3.E-34	pGLH6	NT	N	N
MGG_01609	Aldolase_II PF00596.12 (N. crassa)	6.E-17	pGLH5	9	N	Y
MGG_01660	unique - not in version 6		pGLH248	NA	NA	N
MGG_01764	conserved hypothetical (N. crassa)	9.E-63	pGLH24	3	N	Y
MGG_01863[1]	Peptidase_M28 (PF04389.8)	1.E-54	pGLH115	NT	N	H
MG02066.4[1]	unique - not in version 6		pGLH176	9(+)	N	N
MGG_02073	unique		pGLH249	NA	NA	N
MG02147.4[1]	MGG_07352 - not in version 6 DNA	3.E-07	pGLH251	NA	NA	N
MG02200.4[1]	unique - not in version 6		pGLH178	NA	NA	N
MG02209.4[1]	unique - not in version 6		pGLH253	NA	NA	N
MGG_02234	MGG_08373	5.E-29	pGLH202	7,9	Y	N
MGG_02261[1]	Tannase (PF07519.2)	3.E-76	pGLH133	7,9	N	Y
MG02307.4[1]	unique - not in version 6		pGLH255	NA	NA	N
MGG_02347	DUF1924 (PF09086.2)	7.E-01	pGLH256	NA	NA	Y
MGG_02371	GMC_oxred_C (PF05199.4)	2.E-21	pGLH86	7	Y	Y
MGG_02497	COesterase (PF00135.19)	7.E-62	pGLH85	NT	N	Y
MGG_02502[1]	Glyco_hydro_61 (PF03443.5)	4.E-11	pGLH132	NT	N	N
MGG_02532[1]	Glyco_hydro_7 (PF00840.11)	<E-99	pGLH80	7,9	Y	H
MGG_02546[1]	unique		pGLH257	NA	NA	N

Table 1 (continued)

GENE ID	Sequence similarity	E value	Plasmid	Path	Prot	EST
MG02588.4[1]	unique - not in version 6		pGLH258	NA	NA	N
MGG_02590	MGG_08399	2.E-07	pGLH259	NA	NA	N
MGG_02638[1]	Chitin_bind_3 (PF03067.6)	1.E-01	pGLH260	NA	NA	Y
MGG_02726	Amidase (PF01425.12)	5.E-70	pGLH102	7,9	N	Y
MGG_02819	Metallophos (PF00149.19)	3.E-06	pGLH129	9	N	Y
MGG_02848	conserved hypothetical (N. crassa)	4.E-11	pGLH262	NA	NA	Y
MGG_02853	S1-P1_nuclease (PF02265.7)	1.E-84	pGLH81	5	N	Y
MGG_02915	FAD_binding_4 (PF01565.14)	7.E-30	pGLH82	7,9	N	H
MGG_02989	MGG_08529	3.E-08	pGLH264	NA	NA	N
MGG_03245	Aldose_epim (PF01263.11)	2.E-47	pGLH91	9	N	Y
MGG_03326	conserved hypothetical (N. crassa)	1.E-27	pGLH90	NT	NT	Y
MGG_03346[1]	Asparaginase_2 (PF01112.9)	<E-99	pGLH136	NT	N	N
MGG_03369	Lipocalin_2 (PF08212.3)	3.E-14	pGLH204	7,9	Y	Y
MGG_03374	Cellulase (PF00150.9)	1.E-03	pGLH88	9	N	N
MGG_03457	conserved hypothetical (N. crassa)	2.E-50	pGLH87	5,7	Y	N
MGG_03475[1]	unique		pGLH267	NA	NA	N
MGG_03566[1]	conserved hypothetical (N. crassa)	7.E-10	pGLH135	5,9	N	Y
MGG_03593	conserved hypothetical (N. crassa)	2.E-48	pGLH134	7,9	N	Y
MGG_03671	MGG_08435	2.E-17	pGLH205	9, 9(+)	N	N
MGG_03685	Peptidase_M35 (PF02102.6)	6.E-02	pGLH175	NT	N	Y
MGG_03746	CBM_1 (PF00734.9) xylan esterase	9.E-14	pGLH96	9	Y	Y
MGG_03759	Gpi16 (PF04113.5)	<E-99	pGLH142	7,9	N	Y
MGG_03791	MGG_03806	3.E-11	pGLH269	NA	NA	N
MGG_04015	Glyco_hydro_76 (PF03663.5)	<E-99	pGLH93	NT	N	H
MGG_04206	conserved hypothetical (N. crassa)	5.E-69	pGLH141	NT	N	Y
MGG_04218	unique		pGLH271	NA	NA	N
MG04226.4[1]	unique - not in version 6		pGLH206	9	N	N
MG04303.4[1]	unique - not in version 6		pGLH207	9	N	N
MGG_04309[1]	conserved hypothetical (N. crassa)	6.E-89	pGLH138	5,9	N	N
MG04347.4[1]	unique - not in version 6		pGLH272	NA	NA	N
MGG_04348	Pec_lyase_C (PF00544.10)	1.E-44	pGLH92	3,5	N	Y
MGG_04355	unique		pGLH273	NA	NA	N
MGG_04534	Glyco_hydro_18 (PF00704.19)	1.E-77	pGLH98	5	N	N
MGG_04547	Glyco_hydro_61 (PF03443.5)	6.E-10	pGLH26	9,3	N	H
MGG_04582	conserved hypothetical (N. crassa)	5.E-86	pGLH27	9	N	Y
MGG_04599	conserved hypothetical (N. crassa)	2.E-21	pGLH28	5,7,9	N	Y
MG04754.4[1]	unique - not in version 6		pGLH208	NT	NT	N
MGG_04765	Glyco_hydro_76 (PF03663.5)	1.E-91	pGLH119	NT	N	Y
MGG_04828	conserved hypothetical (N. crassa)	2.E-09	pGLH97	5,7	N	N
MGG_04859	DUF655 (PF04919.3)	2.E-01	pGLH275	NA	NA	N
MGG_04892[1]	unique		pGLH276	NA	NA	N
MGG_04916[1]	unique		pGLH277	NA	NA	N
MGG_04944	conserved hypothetical (N. crassa)	3.E-54	pGLH101	7,9	Y	Y
MGG_04991	conserved hypothetical (N. crassa)	<E-99	pGLH49	9	N	N
MGG_05100	SCP (PF00188.17)	8.E-15	pGLH278	NA	NA	Y
MGG_05127	MGG_10313	2.E-11	pGLH279	NA	NA	N
MGG_05232	conserved hypothetical (N. crassa)	5.E-60	pGLH100	7	Y	Y
MGG_05242	conserved hypothetical (N. crassa)	2.E-35	pGLH99	3,5	Y	N
MGG_05344	Cerato-platanin (PF07249.3)	4.E-77	pGLH59	9	Y	Y
MGG_05364	Glyco_hydro_61 (PF03443.5)	1.E-61	pGLH60	9	N	N
MGG_05389[1]	Kunitz_BPTI (PF00014.14)	5.E-01	pGLH280	NA	NA	N

Table 1 (continued)

GENE ID	Sequence similarity	E value	Plasmid	Path	Prot	EST
MG05403.4[1]	MGG_07352 - not in version 6	6.E-17	pGLH281	NA	NA	N
MGG_05408	unique		pGLH282	NA	NA	N
MGG_05456	CFEM (PF05730.2)	2.E-03	pGLH32	NT	NT	Y
MGG_05483[1]	Fasciclin (PF02469.13)	1.E-23	pGLH61	NT	N	Y
MGG_05504	unique		pGLH146	7	Y	N
MGG_05530[1]	DUF1996 (PF09362.1)	<E-99	pGLH33	3,5	N	N
MGG_05531	CFEM (PF05730.2)	4.E-09	pGLH34	5,9	N	Y
MGG_05632	PBP_GOBP (PF01395.13)	5.E-01	pGLH212	NA	NA	Y
MG05560.5[1]	MGG_07352 - not in version 6	2.E-23	pGLH351	NA	NA	N
MGG_05685	Lectin_leg-like (PF03388.4)	1.E-10	pGLH21	NT	N	Y
MGG_05744[1]	CVNH (PF08881.1)	8.E-06	pGLH213	NT	N	N
MGG_05751	MF_alpha_N (PF05436.2)	7.E-04	pGLH284	NA	NA	N
MGG_05831	MGG_10557	8.E-18	pGLH285	NA	NA	N
MGG_05884	unique		pGLH147	NT	NT	N
MGG_05943	MGG_09826	3.E-24	pGLH183	NT	Y	Y
MGG_05982	unique		pGLH286	NA	NA	Y
MGG_06069[1]	Glyco_hydro_61 (PF03443.5)	3.E-09	pGLH12	9	N	Y
MGG_06103[1]	conserved hypothetical (N. crassa)	6.E-50	pGLH106	9	N	Y
MGG_06116[1]	conserved hypothetical (N. crassa)	1.E-66	pGLH13	NT	N	N
MGG_06206	conserved hypothetical (N. crassa)	7.E-24	pGLH148	7,9	Y	Y
MGG_06224	unique		pGLH149	NT	NT	N
MGG_06225	unique		pGLH214	NT	NT	Y
MGG_06234	DUF1119 (PF06550.2)	7.E-02	pGLH215	7,9	Y	N
MG06253.5[1]	MGG_07352 - not in version 6	2.E-08	pGLH216	3,9	Y	N
MGG_06442	Catalase (PF00199.10)	<E-99	pGLH23	NT	Y	Y
MGG_06538	Bys1 (PF04681.3)	3.E-03	pGLH114	9	Y	Y
MG06592.5[1]	MGG_07352 - not in version 6	6.E-14	pGLH356	NA	NA	N
MG06625.4[1]	unique - not in version 6		pGLH184	NT	N	N
MGG_06665	GASA (PF02704.5)	4.E-02	pGLH287	NA	NA	N
MGG_06746[1]	unique		pGLH150	NT	NT	Y
MGG_06773[1]	Extensin_2 (PF04554.4)	2.E-01	pGLH131	NT	NT	N
MGG_06834	Glyco_hydro_7 (PF00840.11)	0.E + 00	pGLH84	5,7	Y	Y
MGG_06840	Chitosanase (PF07335.2)	3.E-01	pGLH185	7	Y	Y
MGG_06866	conserved hypothetical (N. crassa)	2.E-52	pGLH130	9	N	N
MGG_06906	Peptidase_M14 (PF00246.15)	<E-99	pGLH83	NT	N	Y
MGG_06953	DUF1096 (PF06493.2)	4.E-01	pGLH288	NA	NA	Y
MGG_07005[1]	CFEM (PF05730.2)	7.E-10	pGLH63	9	N	Y
MGG_07016	Lipase_3 (PF01764.16)	1.E-55	pGLH35	9	Y	N
MGG_07050	unique		pGLH289	NA	NA	N
MGG_07100	unique		pGLH151	NT	NT	N
MGG_07234[1]	FKBP_C (PF00254.19)	6.E-48	pGLH94	5,7,9	N	Y
MGG_07246	conserved hypothetical (N. crassa)	2.E-24	pGLH186	7,9	Y	N
MGG_07264	DUF1793 (PF08760.2)	2.E-86	pGLH139	3,5,7	N	Y
MGG_07294	Peptidase_S9 (PF00326.12)	4.E-04	pGLH89	9	Y	H
MGG_07303	unique		pGLH218	3,7	Y	N
MGG_07352	ANATO (PF01821.9)	1.E-01	pGLH352	NA	NA	N
MG07360.4[1]	unique - not in version 6		pGLH152	NT	N	N
MGG_07411[1]	unique		pGLH219	7,9	N	Y
MGG_07424	conserved hypothetical (N. crassa)	2.E-24	pGLH107	9	Y	N
MGG_07520[1]	Glyco_hydro_43 (PF04616.5)	4.E-07	pGLH14	9	N	N
MGG_07554[1]	MGG_07871	6.E-18	pGLH292	NA	NA	N

Table 1 (continued)

GENE ID	Sequence similarity	E value	Plasmid	Path	Prot	EST
MGG_07568	conserved hypothetical (N. crassa)	2.E-11	pGLH15	9	N	N
MGG_07571	conserved hypothetical (N. crassa)	6.E-22	pGLH293	NA	NA	Y
MGG_07575[1]	Glyco_hydro_61 (PF03443.5)	4.E-45	pGLH108	7,9	N	N
MGG_07582[1]	repeat element		pGLH154	7,9	N	Y
MG07588.4[1]	unique - not in version 6 DNA		pGLH188	NA	NA	N
MGG_07591	Baculo_PEP_C (PF04513.3)	7.E-02	pGLH294	NA	NA	N
MGG_07643[1]	conserved hypothetical (N. crassa)	1.E-83	pGLH110	9	N	N
MGG_07644	conserved hypothetical (N. crassa)	1.E-68	pGLH50	9	N	N
MGG_07646	Glyco_hydro_67M (PF07488.3)	<E-99	pGLH16	NT	Y	N
MGG_07656[1]	Chitosanase (PF07335.2)	<E-99	pGLH17	NA	NA	N
MGG_07686[1]	Glyco_hydro_61 (PF03443.5)	4.E-10	pGLH18	9	Y	Y
MGG_07699	MGG_04546	2.E-08	pGLH155	7,9	N	N
MGG_07715	Glyco_hydro_16 (PF00722.12)	5.E-04	pGLH52	9	Y	N
MGG_07749	conserved hypothetical (N. crassa)	1.E-16	pGLH295	NA	NA	N
MGG_07767[1]	conserved hypothetical (N. crassa)	9.E-89	pGLH20	9	Y	N
MGG_07786	conserved hypothetical (N. crassa)	1.E-28	pGLH53	9	N	N
MGG_07810	duplicate gene w/MGG_13868		pGLH296	NA	NA	N
MGG_07834	zf-C2H2 (PF00096.17)	7.E-01	pGLH297	NA	NA	N
MGG_07900	unique		pGLH299	NA	NA	N
MGG_07901	conserved hypothetical (N. crassa)	8.E-32	pGLH30	9	Y	Y
MGG_07965	Peptidase_S8 (PF00082.13)	5.E-59	pGLH55	0,3	Y	H
MG07978.4[1]	unique - not in version 6		pGLH300	NA	NA	N
MGG_07986	unique		pGLH222	5,9	N	N
MGG_07993	unique		pGLH301	NA	NA	N
MGG_08041	Peptidase_M43 (PF05572.4)	7.E-05	pGLH51	7,9	Y	Y
MGG_08054	Glyco_hydro_18 (PF00704.19)	9.E-42	pGLH118	3,7,9	Y	Y
MGG_08080	BIG1 (PF08319.2)	4.E-47	pGLH25	9	N	Y
MGG_08086	Myc_N (PF01056.9)	7.E-01	pGLH302	NA	NA	N
MGG_08096	EMP24_GP25L (PF01105.15)	5.E-10	pGLH116	NT	N	Y
MGG_08200[1]	WSC (PF01822.10)	1.E-25	pGLH109	NT	N	H
MGG_08291	conserved hypothetical (N. crassa)	3.E-21	pGLH303	NA	NA	Y
MGG_08376	unique		pGLH304	NA	NA	N
MG08392.4[1]	unique - not in version 6		pGLH157	7,9	N	N
MG08394.4[1]	MGG_07352 - not in version 6	2.E-02	pGLH305	NA	NA	N
MGG_08399	CVNH (PF08881.1)	5.E-01	pGLH306	NA	NA	N
MGG_08407	unique		pGLH224	7,9	Y	N
MGG_08424	Glyco_hydro_11 (PF00457.8)	<E-99	pGLH31	NT	NT	H
MGG_08428	unique		pGLH189	NA	NA	N
MGG_08431	Dioxygenase_C (PF00775.12)	3.E-07	pGLH57	9	N	N
MGG_08432	unique		pGLH190	NA	NA	N
MGG_08435	MGG_03671	4.E-19	pGLH191	7	N	N
MGG_08451	ANATO (PF01821.9)	5.E-03	pGLH225	3,5	Y	Y
MGG_08469	unique		pGLH158	9	Y	N
MGG_08481	CMV_US (PF08001.2)	9.E-01	pGLH307	NA	NA	N
MGG_08482	unique		pGLH159	9	N	N
MGG_08543	MGG_08796	7.E-35	pGLH308	NA	NA	Y
MGG_08546	unique		pGLH309	NA	NA	N
MG08603.4[1]	unique - not in version 6		pGLH310	NA	NA	N
MGG_08644	conserved hypothetical (N. crassa)	6.E-17	pGLH126	9	N	N
MG08717.4[1]	unique - not in version 6		pGLH311	NA	NA	N
MGG_08720	repeat element		pGLH312	NA	NA	Y

Table 1 (continued)

GENE ID	Sequence similarity	E value	Plasmid	Path	Prot	EST
MGG_08787[1]	unique		pGLH313	NA	NA	N
MGG_08797	unique		pGLH161	9	Y	N
MGG_08817	Toxin_18 (PF08087.2)	3.E-01	pGLH162	7,9	Y	Y
MGG_08823	Glyco_hydro_16 (PF00722.12)	7.E-05	pGLH1	9	N	Y
MGG_08938[1]	Glyco_hydro_28 (PF00295.8)	5.E-40	pGLH144	9	N	N
MGG_08940	unique		pGLH163	NA	NA	N
MGG_08962[1]	MGG_08373	7.E-48	pGLH226	7,9	N	N
MGG_08966	Peptidase_S8 (PF00082.13)	7.E-61	pGLH143	NA	NA	H
MG08991.4[1]	unique - not in version 6		pGLH315	NA	NA	N
MGG_09032	Peptidase_A4 (PF01828.8)	6.E-18	pGLH70	5,9	N	N
MGG_09134[1]	Hydrophobin_2 (PF06766.2)	7.E-34	pGLH227	7	N	Y
MG09140.4[1]	unique - not in version 6		pGLH165	5,9	N	N
MG09155.4[1]	MGG_07352 - not in version 6	1.E-07	pGLH316	NA	NA	N
MGG_09160[1]	Glyco_hydro_3 (PF00933.12)	<E-99	pGLH127	NA	NA	H
MGG_09237	conserved hypothetical (N. crassa)	1.E-49	pGLH62	9	N	Y
MGG_09271[1]	conserved hypothetical (N. crassa)	2.E-84	pGLH71	9	N	Y
MGG_09351[1]	Asp (PF00026.14)	6.E-79	pGLH122	3,5	N	H
MGG_09378	MGG_14502	9.E-10	pGLH317	NA	NA	Y
MGG_09412	conserved hypothetical (N. crassa)	4.E-55	pGLH64	9	N	Y
MGG_09425	unique		pGLH318	NA	NA	N
MGG_09452[1]	MGG_07810	3.E-19	pGLH229	9	N	N
MGG_09465	conserved hypothetical (N. crassa)	1.E-22	pGLH41	9	N	Y
MGG_09474	unique		pGLH319	NA	NA	Y
MGG_09576	conserved hypothetical (N. crassa)	6.E-24	pGLH42	9	N	N
MG09651.4[1]	unique - not in version 6		pGLH320	NA	NA	N
MG09652.4[1]	unique - not in version 6		pGLH167	7,9	N	N
MGG_09675	unique		pGLH168	5,7,9	N	N
MGG_09693	conserved hypothetical (N. crassa)	9.E-12	pGLH194	7,9	N	H
MGG_09726	Glyco_hydro_53 (PF07745.4)	<E-99	pGLH43	9	Y	N
MGG_09803	unique		pGLH195	5,7,9	N	N
MGG_09804[1]	conserved hypothetical (N. crassa)	2.E-18	pGLH45	NT	N	Y
MGG_09826	MGG_05943	8.E-20	pGLH196	5,7	N	H
MGG_09842	conserved hypothetical (N. crassa)	4.E-28	pGLH231	1,7	Y	Y
MGG_09861[1]	Glyco_hydro_16 (PF00722.12)	1.E-08	pGLH68	NT	N	Y
MGG_09918[1]	Glyco_hydro_16 (PF00722.12)	3.E-65	pGLH67	9	N	Y
MGG_09998	unique		pGLH197	7,9	Y	Y
MGG_10021[1]	unique		pGLH232	7,9	N	N
MGG_10090[1]	unique		pGLH233	NA	NA	N
MG10100.4[1]	MGG_07352 - not in version 6 DNA	4.E-16	pGLH324	NA	NA	N
MGG_10234	unique		pGLH325	NA	NA	N
MGG_10275[1]	Metallophos (PF00149.19)	3.E-11	pGLH72	NA	NA	N
MGG_10315	Hydrophobin (PF01185.9)	1.E-31	pGLH234	7,9	Y	Y
MGG_10335	DUF1212 (PF06738.3)	4.E-01	pGLH327	NA	NA	N
MGG_10361	unique		pGLH38	7,9	N	N
MGG_10424	DPBB_1 (PF03330.9)	2.E-04	pGLH39	5,9	Y	N
MGG_10449	Peptidase_S8 (PF00082.13)	2.E-69	pGLH46	NA	NA	H
MGG_10456	unique		pGLH198	5,7	N	Y
MGG_10490	Peptidase_C13 (PF01650.9)	3.E-18	pGLH47	7,9	N	Y
MGG_10531	unique		pGLH331	NA	NA	N
MGG_10533	Arginase (PF00491.12)	2.E-47	pGLH73	9	N	H
MGG_10618	acetylxylan esterase	0.E + 00	pGLH22	9	N	Y

Table 1 (continued)

GENE ID	Sequence similarity	E value	Plasmid	Path	Prot	EST
MG10622.4[1]	unique - not in version 6		pGLH173	5,9	N	N
MG10732.5[1]	MGG_07352 - not in version 6	1.E-06	pGLH237	5,7,9	Y	N
MGG_10744	Beta-lactamase (PF00144.15)	1.E-17	pGLH48	NA	NA	N
MG10771.4[1]	unique - not in version 6		pGLH332	NA	NA	N
MGG_10780	MGG_13755	2.E-24	pGLH333	NA	NA	N
MGG_10796	unique		pGLH334	NA	NA	N
MGG_10824	conserved hypothetical (N. crassa)	6.E-16	pGLH3	9	N	Y
MGG_10914	BmKX (PF09132.1)	4.E-01	pGLH239	7,9	Y	N
MG10942.4[1]	MGG_07352 - not in version 6	1.E-04	pGLH354	NA	NA	Y
MG10967.4[1]	unique - not in version 6 DNA		pGLH335	NA	NA	N
MGG_11072	unique		pGLH336	NA	NA	N
MGG_11335	WSC (PF01822.10) (N. crassa)	<E-99	pGLH2	9	N	Y
MGG_11553	protein similar to Cel74a	0.E + 00	pGLH123	3,5	Y	Y
MG11556.4[1]	unique - not in version 6		pGLH211	9	Y	N
MGG_11610	unique		pGLH243	NA	NA	N
MGG_12025[1]	Aldose 1-epimerase (N. crassa)	<E-99	pGLH65	3,9	N	H
MG12674.4[1]	unique - not in version 6		pGLH179	NA	NA	N
MGG_12942	unique		pGLH209	7,9	Y	Y
MGG_13008[1]	unique		pGLH230	5,9	Y	N
MG13022.5[1]	unique - not in version 6		pGLH210	9	N	N
MG13089.5[1]	MGG_07352 - not in version 6	2.E-05	pGLH353	NA	NA	N
MG13093.5[1]	unique - not in version 6		pGLH145	9	N	N
MG13132.5[1]	unique - not in version 6		pGLH283	NA	NA	N
MG13351.5[1]	unique - not in version 6		pGLH193	NT	NT	N
MG13357.5[1]	MGG_07352 - not in version 6	3.E-12	pGLH355	NA	NA	N
MG13601.5[1]	MGG_07352 - not in version 6	1.E-18	pGLH350	NA	NA	N
MGG_13654[1]	conserved hypothetical (N. crassa)	7.E-10	pGLH44	9	N	Y
MG13701.5[1]	unique - not in version 6		pGLH201	3,5,9	N	N
MG13911.5[1]	unique - not in version 6		pGLH156	9	N	N
MG13976.5[1]	unique - not in version 6		pGLH192	9	N	N
MGG_14002[1]	conserved hypothetical (N. crassa)	<E-99	pGLH120	NT	NT	H
MG14065.5[1]	unique - not in version 6		pGLH166	NT	NT	N
MGG_14134	Beta-lactamase (PF00144.15)	1.E-17	pGLH40	NA	NA	N
MG14159.5[1]	unique - not in version 6		pGLH169	5,9	N	N
MGG_14212	unique		pGLH235	7	Y	N
MG14383.5[1]	unique - not in version 6		pGLH180	NA	NA	N
MGG_14600	unique		pGLH177	NT	N	N
MGG_14652	MGG_11362	2.E-04	pGLH246	NA	NA	N
MGG_14796	acid protease	<E-99	pGLH7	9	N	Y
MGG_14926	conserved hypothetical (N. crassa)	1.E-06	pGLH199	9	Y	N
MGG_15084	unique		pGLH291	NA	NA	N
MGG_15443	unique		pGLH171	5,9	Y	N
MGS0011[1]	unique - not in version 6 DNA		pGLH240	9	Y	N
MGS1422[1]	unique - not in version 6		pGLH174	3,5	N	N
MGS1439[1]	unique - not in version 6		pGLH238	9	Y	N

[1] Gene not predicted or alternative annotation in Version 6 of the genome annotation. See text for description of column headings. Full version of Table with primer sequences are available at http://www.mgosdb.org

in the *M. grisea* EST databases, and homology to known proteins were used in choosing these initial genes for further analysis.

The 299 cloned genes are listed in order of Gene ID number based on the Broad Institute version 6 gene model (first column, Table 1). A number of genes amplified in our study were predicted based on earlier gene models or manual annotation and differ from the version 6 annotation. These 116 genes are indicated by a superscript 1 (^{1}) following the gene ID number. There are 51 genes that are no longer predicted in version 6 that were predicted in previous versions of the genome annotation. These are indicated by their Gene ID of the previous genome release. Since many of the secreted proteins represent genes that lack homologs in other species, annotation in the absence of cDNA information is challenging. Many of the unique genes predicted to encode small proteins ($<$ 100 amino acids) were dropped from later versions of the genome annotation. The primer sequences used to amplify the genes are shown in an on-line supplementary version of the Table to allow readers to assess the genes predictions (See Table 1 legend). Although some incorrect gene models generating incorrect coding regions or preventing proper fusions to the his-6 tag are present, in the majority of cases the amplified regions we cloned likely produce authentic gene products.

The annotations provided represent either the best PFAM domain (Finn, et al., 2008) matches or the best hits to *Neurospora crassa* genes of unknown function (55 genes) and these were listed as conserved hypotheticals if a strong match to a PFAM domain was not found. A number of genes were found to match only other genes in the *M. oryzae* genome (32 genes) and the similarity to an *M. oryzae* paralog listed is shown. The e-value for the match is provided in the third column of the table. There were 93 genes in the set that were predicted to be unique in the *M. oryzae* genome.

The name of the expression plasmid containing the amplified gene fragment is indicated in the fourth column. These plasmids can be used to transform fungi and contain *E. coli* and *S. cerevisiae* replication origins, along with hygromycin resistance for selection in filamentous fungi. The expression of the genes is driven by the *M. oryzae* ribosomal protein 27 promoter as described (Lu et al., 2004).

Of the 299 constructs, 198 were transformed into *M. oryzae*. In most cases, three independent transformants were stored as permanent stocks. Of the 154 transformed lines that were tested for pathogenicity, only one showed a complete loss of pathogenesis., Constitutive expression of MGG_07965 was confirmed by purification of the protein from the pGLH55 transformed *M. grisea* culture filtrates. These strains produced few appressoria than wild type (70–15) and the purified protease inhibited appressorium formation of wild type strains (not shown).

The pathogenicity rating was determined for one to several independent transformants. Where ratings varied between isolates, more than one rating is given (5th column, Table 1). The wild type was given the rank of 9 and the absense of visible symptoms was rated 0. The rankings were done on the susceptible cultivar M202. The intermediate ranking indicates the number and size of lesions relative to wild type. A rating of 1–2 was given for flecking or pinpoint. Larger lesions were given a higher rating. Individual isolates could give multiple types of lesions and vary in the

number of lesions and the ratings were assigned to reflect overall disease levels but are not meant to be interpreted as rigorous as only one to three pathogenicity tests were performed with each isolate. In just three cases, there was an overall impression of stronger aggressiveness of the transformed lines relative to wild type and these were ranked as 9 (+) in Table 1. In many cases, there was variation between independent lines. When multiple rankings were found, they were represented with the rankings for each assay separated by a comma. For example, if some isolates gave a rating of 7 and another a rank of 9, the overall pathogenicity rating (Path) in Table 1 is given as 7, 9.

Sixty-three lineages produced symptoms indistinguishable from wild type, whereas twenty-nine lineages produced symptoms that were less severe than wild type. The remaining lineages displayed variation between the individual transformed strains with at least one isolate being judged as similar to the 70–15 control. In general, reproducibility on retesting was good, although some variation in rankings was observed. Strains are available for further testing and analysis.

Forty-four of the transformed lines were not tested for pathogenicity (NT). Plasmids that were not transformed into *M. oryzae* (100) are indicated as not available (NA) in the Path column and protein expression could not be assessed for these (NA, 6th column). Sixty-one lineages expressed protein at levels that could be detected from 1 ml culture filtrates while protein was not detected in 120 lineages. Thus, approximately 33% of tested, transformed lines produced high levels of proteins. Other transformed lineages have not yet been confirmed for protein secretion. The 7th column notes if an EST was found for the gene (Y) or not (N) or if an EST with homology to the gene was found (H) based on homology searches of the EST database aligned in the version 6 genome annotation.

Proteins were purified at larger scale (400 ml) for testing on wounded rice leaves. In most cases no reaction was observed. In ~5% of cases, some level of chlorosis or necrosis was often observed. However, these symptoms were variable and the level of response was observed to vary with the age of the leaf and even the position on the leaf. We therefore concluded that most proteins had none or very weak activity in inducing macroscopic symptoms on the M202 susceptible cultivar.

4 Conclusions

A significant effort was made to examine a non-biased sampling of putative secreted proteins from *M. oryzae*. Overall, very few, if any, of the proteins examined acted as microbial associated molecular patterns (PAMPs) that triggered overt defense responses. We conclude that a relatively small fraction of secreted proteins from *M. oryzae* serve as PAMPs for rice cultivar M202.

Further analysis is needed to determine if the transformed lines displaying altered levels of aggressiveness are dependent on expression of the transgenes. We found that 16 of the 94 lineages (17%) that were tested for pathogenity but did not express protein were found to have reduced aggressiveness. It is possible that low levels of proteins were produced or that proteins were membrane or cell wall associated and

not free in the culture filtrates. We found that 14 of the 58 lineages (24%) expressing protein displayed reduced aggressiveness.

Here we have primarily examined if the constitutive expression of these genes would reduce aggressiveness of the isolates. Alternative testing of transformed lineages on resistant cultivars would be of interest to determine if constitutive expression of the genes would enhance aggressiveness. There appeared to be three lineages (MGG_01009, MGG_03671, and MG02066.4) that displayed enhanced aggressiveness relative to the 70–15 parent on M202. MGG_03671 has homology to MGG_08435, however, the MGG_08435 lineage displayed slightly reduced aggressiveness.

In addition to testing proteins for induction of symptoms on rice, it will be of interest to test them for the ability to suppress defense responses induced by known elicitors. In addition to testing wounded rice leaves, it will also be important to test proteins for non-specific elicitor activity on non-hosts, such as tobacco. Although constitutive expression of the genes in *M. oryzae* was important to obtain experimental support that these were secreted proteins, overexpression in better defined expression systems for recovery of higher levels of purified proteins is likely to be required to allow thorough characterization of protein activities.

The plasmids and transformed isolates are available for use of the broader community. Strains and other resources have been used to characterize the role of cell wall degrading enzymes (Zhou et al., 2008), putative virulence factors (Jeong et al., 2007), and to help annotate the Magnaporthe genome (Soderlund et al., 2006).

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Part II
Host Defense Genetics, Genomics, Molecular Biology

Functional and Evolutionary Analysis of the *Pi2/9* Locus in Rice

Bo Zhou and Guo-Liang Wang

Abstract The *Pi2/9* locus has been extensively used for effective resistance to the devastating rice blast disease caused by the fungus *Magnaporthe oryza* in rice breeding program for long time. This locus has been found to harbor at least 6 resistance genes [*Pi9*, *Pi2*, *Piz-t*, *Piz*, *Pigm(t)*, and *Pi40(t)*], which confer broad-spectrum resistance against different sets of *M. oryzae* isolates. We have successfully characterized three of them (*Pi9*, *Pi2*, and *Piz-t*) using multi-faceted approaches. All the three genes encode proteins with a nucleotide-binding site (NBS) and leucine-rich repeat (LRR) domain and belong to a member of a gene cluster comprising of multiple gene members in each resistant cultivar. All the three resistance genes share extreme sequence similarity to each other while the LRR domains possess most of their sequence variants. Strikingly, the total eight amino acid differences that distinguish the Pi2 from the Piz-t were exclusively confined within three consecutive LRRs, indicating that the LRR domain plays a major role in determination of their resistance specificities. Intra- and inter-haplotype analysis of the *Pi2/9* locus from 5 different rice haplotypes revealed that an obvious orthologous relationship had been maintained at the *Pi2/9* locus among haplotypes. The paralogues, contrariwise, show significant sequence divergence within each haplotype. However, the finding that all the NBS-LRR homologues at the *Pi2/9* locus utilize the same intron phase 2 suggested that the *Pi2/9* locus might be evolved from a progenitor locus that was consisted of a single NBS-LRR gene with the same intron phase. The cloning of the three resistance genes and evolutionary analysis of the *Pi2/9* locus provided insight into the understanding of the mechanism underlying the broad-spectrum resistance and its evolution.

Keywords Resistance genes · Evolution · *Oryza sativa* · Gene cluster

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1 Introduction

To prevent the invasion of the pathogen, plant has evolved a continuum of layered defenses. The plant's defensive response is controlled by its genetics and can be achieved at both the species and cultivar level (Da Cunha et al. 2007). A central component of plant resistance response is the hypersensitive response (HR), which is occurred in cultivar resistance and activated by gene-for-gene recognition of a pathogenic determinant by a host encoded resistance (R) protein. The overwhelming majority of plant *R* genes encode the protein with a nucleotide-binding-site (NBS) and C-terminal leucine-rich-repeat (LRR) domain. Based on their amino-terminal sequence, the NBS-LRR-type *R* genes can be divided into two major subfamilies (Bai et al., 2002, Meyers et al., 2003). One subfamily is consisted of TIR-NBS-LRR genes characteristic of the TIR domain homologous to the *Drosophila* Toll and mammalian Interleukin-1 receptor (Hammond-Kosack & Jones 1997), and the other subfamily is consisted of CC-NBS-LRR genes characteristic of the coiled-coil (CC) motif (Pan et al. 2000). Interestingly, no TIR-NBS-LRR genes have yet been identified in rice as well as in other cereals, suggesting a different *R* gene repertoire in cereals from the one in *Arabidopsis* as well as in other dicot plants (Bai et al. 2002; Meyers et al. 2003).

Rice blast, caused by the fungal pathogen *Magnaporthe oryzae*, is the most destructive fungal disease of rice and causes tremendous rice yield loss in the world every year (Talbot 2003). Use of host resistance has been proved to be the most effective and economic way in preventing the rice blast disease. To date, over 50 rice blast resistance genes have been genetically analyzed and mapped (Liu et al. 2007). Eight of them (*Pib*, *Pi-ta*, *Pi-d2*, *Pi2*, *Pi9*, *Piz-t*, *Pi36* and *Pi37*) have been successfully cloned (Table 1). Except for *Pi-d2*, which encodes a protein homologous to B-lectin protein-like kinase, the other seven *R* genes encode NBS-LRR proteins. Nevertheless, only one of six cloned rice bacterial blight resistance genes encode a NBS-LRR protein (Dai et al. 2007). The finding that most of the cloned rice blast *R* genes encode NBS-LRR type proteins may suggest that NBS-LRR type genes constitute the major repertoire of rice blast resistance genes.

With respect to the genomic organization of *R* genes in rice, most of them are located in a genetic complex region, in which multiple sequence-related genes are

Table 1 Summary of cloned rice blast resistance genes

Gene	Characteristics	Chromosome location	Complex locus	Reference
Pib	NBS-LRR	2	Yes	Wang et al. 1999
Pi-ta	NBS-LRR	12	No	Bryan et al. 2000
Pi-d2	B-lectin receptor kinase	8	No	Chen et al. 2006
Pi9	NBS-LRR	6	Yes	Qu et al. 2006
Pi2	NBS-LRR	6	Yes	Zhou et al. 2006
Piz-t	NBS-LRR	6	Yes	Zhou et al. 2006
Pi36	NBS-LRR	8	Yes	Liu et al. 2007
Pi37	NBS-LRR	1	Yes	Lin et al. 2007

tandem arrayed within a single locus (Table 1). Two *Pib* homologues were identified at the *Pib* locus (Wang et al. 1999). The *Pi36* and *Pi37* loci were consisted of three and four NBS-LRR genes, respectively, in Nipponbare (Liu et al. 2007; Lin et al. 2007). The *Pi2/9* locus was comprised of 7 to 9 NBS-LRR genes in different rice cultivars (Zhou et al. 2007). The *Pi-ta* and *Pi-d2*, on the contrary, contain a single *R* gene at each locus (Bryan et al. 2000; Chen et al. 2006).

2 The *PI2/9* Locus

Until now, there are at least six rice blast resistance genes are mapped at the *Pi2/9* locus, which is proximal to the centromere of rice chromosome 6. They are *Pi2*, *Pi9*, *Piz-t*, *Piz*, *Pigm(t)*, and *Pi40(t)* (Liu et al. 2002; Hayashi et al. 2004; Deng et al. 2006; Jeung et al. 2007). These six resistance genes all confer broad spectrum resistance to *M. oryzae* (Liu et al. 2002; Kato et al. 2004; Deng et al. 2006; Jeung et al. 2007). Moreover, their resistance specificities were found different to each other. Based on their origin, these six resistance genes each was identified in different donor rice cultivar as well as in wild rice species. The *Pi2* in the isogenic lines C101A51 was introduced from *indica* rice cultivar 5173 while the *Piz-t* gene in the rice cultivar Toride 1 was from another *indica* rice cultivar TKM1 (Nagai et al. 1970; Mackill and Bonman 1992). The *Piz* gene was originally reported in the US cultivar Zenith (Kiyosawa 1967). The *Pi9* gene in the isogenic line 75-1-127 was introduced from *Oryza minuta*, a tetraploid wild species of the *Oryza* genus (Amante-Bordeos et al. 1992). The *Pigm(t)* was originally identified in an *indica* rice variety in China (Deng et al. 2006). The *Pi40(t)* was identified in the introgression line IR65482-4-136-2-2 that had inherited the resistance from an EE genome of the wild species, *O. australiensis* (Jeung et al. 2007). The *Pi2/9* locus clustered with multiple *R* alleles with overlapping but different resistance specificities provides a unique genetic locus to understand the mechanism of the broad spectrum resistance to rice blast.

3 Cloning of Three *R* Genes (*PI9, PI2*, AND *PIZ-T*)

We employed a map-based cloning strategy to isolate both *Pi2* and *Pi9* genes. The *Pi9* gene was embedded in the 76-kb genomic region clustered with six NBS-LRR genes (Qu et al. 2006). Analysis of *Pi9* suppressive mutants and complementation test by using TAC vector with large genomic DNA fragment further delimited the *Pi9* gene to the genomic region containing *Nbs2-Pi9* and *Nbs3-Pi9*. Gene complementation with respective *Nbs2-Pi9* and *Nbs3-Pi9* finally determined that *Nbs2-Pi9* is the *Pi9* gene. Resistance reaction assay of the transgenic *Pi9* plants using a diverse set of isolates of *M. oryzae* found that the transgenic plants had the same resistance spectrum as the *Pi9* isogenic line. The *Pi2* gene was firstly mapped between the

two markers Nbs1-Pi9 and Nbs4-Pi9, which were developed based on the genomic sequence of the *Pi9* region. Complete sequence of the *Pi2* locus helped us to narrow down the *Pi2* candidate to the genomic region containing 6 NBS-LRR genes (Zhou et al. 2006). Two susceptible mutants, in which a two-nucleotide deletion in the coding sequence of *Nbs4-Pi2* was identified, which facilitated the further delimitation of the *Pi2* gene. Gene complementation test and resistance reaction of the derived transgenic plants finally confirmed that *Nbs4-Pi2* is the *Pi2* gene and that its transgenic plants had the same resistance spectrum as the *Pi2* donor lines. Based on the comparison of the NBS-LRR genes between *Pi2* and *Piz-t* locus, we found that the *Piz-t* locus is almost identical to the *Pi2* locus. However, *Nbs4-Piz-t* had 11 nucleotide sequence variation comparing to *Nbs4-Pi2*, which resulted in a total of 8 amino acid differences between their encoded products (Zhou et al. 2006). The sequence polymorphism between *Nbs4-Piz-t* and *Nbs4-Pi2* prompted us to speculate that *Nbs4-Piz-t* might be the *Piz-t* candidate, which was further confirmed by gene complementation test and resistance spectrum analysis of the transgenic plants.

Pi2, *Pi9* and *Piz-t* encode NBS-LRR proteins, which belong to the major nT-NBS-LRR gene family in rice (Qu et al. 2006; Zhou et al. 2006). A conserved nT motif was identified at their N-terminal region and 17 imperfect LRRs were clustered at their C-terminal region; the NBS domain contained all the conserved motifs (Kinase 1a, P-loop, Kinase 2, RNBS-B, and GLPL) was situated in the middle. Comparative analysis among these three resistance genes revealed that they share extreme sequence identity to each other. However, the LRR region contains the most sequence variants, indicating that the LRR region plays a major role in the determination of the resistance specificity. For example, the total 8 amino acid differences between Pi2 and Piz-t were exclusively confined into 3 consecutive LRRs. Pi9 has a total of 43 and 46 amino acid changes from Pi2 and Piz-t, respectively. Of these sequence changes, 34 and 37 variants from the pairs of Pi2/Pi9 and Piz-t/Pi9, respectively, were confined within the LRR region (Qu et al. 2006; Zhou et al. 2006). Among the 8 amino acid changes between Pi2 and Piz-t, only one amino acid difference (arginine-838 in Pi2 versus serine-839 in Piz-t) is situated in the motif of xxLxLxx, which is predicted to be involved in recognition of the cognate pathogen effectors. However, both recombinant genes derived from reciprocal exchange of this amino acid change between Pi2 and Piz-t, designated Nbs4-Pi2^{838S} and Nbs4-Piz-t^{839R}, were found to lose the recognition ability to either AvrPi2 or AvrPiz-t verified with the transgenic rice plants expressing the recombinant genes (Zhou et al. 2006). This result demonstrated that the exchange of the single amino acid in xxLxLxx motif is not sufficient to switch the resistance specificity.

Different from the *Pib* gene, whose expression was induced by some altered environmental conditions, all the three *R* genes at the *Pi2/9* locus was found expressed constitutively (Qu et al. 2006; Zhou et al. 2006). They showed a similar expression pattern with other three cloned rice blast *R* genes (*Pi-ta*, *Pi36* and *Pi37*), suggesting that most of NBS-LRR type *R* genes are likely expressed constitutively.

4 *PI2, PI9, PIZ-T*, and *PIZ* Are Allelic to Each Other

Cloning of *Pi2*, *Pi9*, and *Piz-t* and sequence determination of their respective locus revealed that they are indeed allelic to each other at the locus level. However, we found that *Pi2* and *Piz-t* were situated at the exactly same genomic location within the NBS-LRR gene cluster, indicating that they are true alleles to each other (Fig. 1). Nevertheless, the *Pi9* gene appears situated in different location from both *Pi2* and *Piz-t*, suggesting that *Pi9* and *Pi2/Piz-t* each has inherited from one of the duplicated genes from their common ancestor. If this is the case, it will be more reasonable to consider the *Pi9* as the paralogue to both *Pi2* and *Piz-t*. Interestingly, we found that the *Piz* locus from the *Piz*-carrying rice cultivar Zenith showed a similar genomic organization as the *Pi9* locus since it contains *Nbs2-Pi9*'s orthologue at the interval region between *Nbs1-Pi9* and *Nbs3-Pi9* (Fig. 1). The deduced protein of *Nbs3-Piz* has only 11 amino acid differences from its orthologue *Nbs3-Pi9*, i.e. the *Pi9* gene (unpublished data).

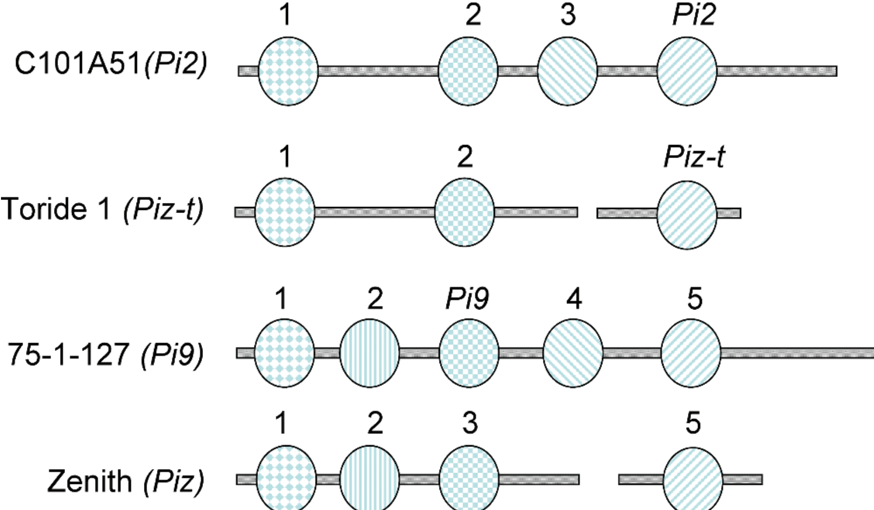

Fig. 1 Schematic genomic organization of the NBS-LRR genes at the *Pi2/9* locus in four different resistance rice cultivars. The *gray* bar represents the genomic sequence of the *Pi2/9* locus. The NBS-LRR genes are shown with ovals with different color representing different orthologues. The genes are named numerically according to their order in the cluster and the ones not listed due to undetermined sequence indicated by gaps between *gray* bars. The genes are not listed in scale to make good alignment of the orthologues. The rice cultivars or isogenic lines are listed at the left side and their corresponding *R* gene is in parenthesis

5 The Evolution and Dynamics of the *PI2/9* Locus

To investigate the organization and evolutionary mechanism of the *Pi2/9* locus, we sequenced five different rice cultivars including both *Pi2*- and *Pi9*-isogenic lines, representing the *Pi2/9* locus in two rice subspecies and both resistance and

susceptible rice cultivars. The *Pi2/9* locus in each cultivar is consisted of multiple NBS-LRR genes flanked by a gene homologous to the nitrated induced protein gene at the distal side and by a gene homologous to the protein kinase gene at proximal side to the centromere of chromosome 6 (Zhou et al. 2007). A total of 9 and 7 NBS-LRR genes were identified at the *Pi2* locus and at the *Pi2/9* locus in Nipponbare, a susceptible *japonica* rice cultivar, respectively. Nevertheless, the total number of the NBS-LRR genes at the *Pi2/9* locus in other three cultivars [75-1-127 (the *Pi9*-isogenic line), CO39, and 9311] were not completely determined due to the sequence gaps. Based on the known sequence, a total of 9, 4, and 6 NBS-LRR genes were identified at the *Pi2/9* locus in 75-1-127, CO39, and 9311, respectively. It seems that the *Pi9* locus contains the most number of NBS-LRR genes since it has two additional orphan members which are not present in other four cultivars.

The NBS-LRR paralogues share significant sequence variation to each other at the *Pi2/9* locus within each haplotype. For example, the sequence similarity in nucleotide of pairs of paralogues ranges from 58.1% to 96.3% at the *Pi2* locus (Zhou et al. 2007). A similar feature of the sequence divergence among the paralogues was also observed in other four haplotypes. A striking feature observed at the *Pi2/9* locus is the asymmetric sequence divergence between the coding sequence and noncoding sequence. Both 5′ regulatory region and first intron are more divergent of their sequence and size than the coding sequence. For example, *Nbs2-Pi2*, *Nbs4-Pi2*, and *Nbs6-Pi2* at the *Pi2* locus share as high as 96% nucleotide sequence similarity in their coding region to each other whereas they do not share any sequence identity in their 5′ regulatory sequence and in their first introns. However, we found that all the NBS-LRR genes in five haplotypes utilized the same phase-2 intron which was highly conserved in the evolution of NBS-LRR genes (Meyers et al. 2003).

In contrary to the significant sequence divergence among the paralogues observed within each haplotype, an obvious orthologous relationship among different haplotypes was found at the *Pi2/9* locus. The NBS-LRR genes at the same genomic location in different haplotype exhibit extreme sequence identity to each other and they are much similar to each other than themselves to their paralogues within each haplotype. The obvious orthologous relationship maintained at the *Pi2/9* locus might attribute to the less frequency of the inter- and intra-genic sequence exchange during its evolution. It has been demonstrated that the sequence divergence at the 5′ portion of the gene correlates negatively with the frequency of the crossover between the sister chromosomes in meiosis (Duret et al. 1995; Carvalho & Clark 1999; Comeron and Kreitman 2000). In this context, we proposed that the sequence and size divergence of the NBS-LRR genes at the *Pi2/9* locus might play a recombination suppressor which largely restrained the inter- and intra-genic recombination.

In order to further investigate the genetic events involved in the evolution of the *Pi2/9* locus, we have completely sequenced the *Pi2/9* locus in four different wild rice species (*O. nivara*, *O. punctata*, *O. Officinalis*, *and O. minuta*). It has been found that most of the NBS-LRR homologues identified in different wild rice species conserved the same feature with respect to the intron phase and positions, which further suggested that the *Pi2/9* locus had been evolved from a progenitor consisted

of a single NBS-LRR gene with the same intron phase and position. However, we observed that the *Pi2/9* locus is more dynamic within and among different wild rice species than the one within the cultivated rice haplotypes. Each wild species has a different copy number of the NBS-LRR genes, some of which were even located outside of the protein kinase gene. Moreover, the accumulation of the sequence mutation, insertion and deletion that made the sequence unable to encode a full length protein was identified in most of the NBS-LRR homologues in the four wild rice species. In contrary to the finding within different cultivated rice haplotypes, a weak orthologous relationship was present in different wild rice species due to the sequence inversion, translocation and other genetic events. Even if the orthologues were identified in different species, the sequence similarity was only observed in their coding sequence but not in their non-coding sequence. The finding that the divergence of the intron sequence and size was significantly involved in the evolution of the *Pi2/9* locus may provide a novel insight into the understanding of the evolutionary mechanism of the NBS-LRR gene loci.

6 Cloning of the *AVRPI2* and *AVRPIZ-T* Genes

It should be intriguing to isolate the corresponding avirulence genes to the resistance genes at the *Pi2/9* locus, which should provide valuable insight into understanding the mechanism of broad spectrum resistance mediated by these three resistance genes. We deployed a map-based cloning strategy to isolate both *AvrPiz-t* and *AvrPi2* using a same F_1 genetic population derived from the cross between 81278ZB15 and GUY11. The strain 81278Zb15 is avirulent to both *Pi2* and *Piz-t* whereas GUY11 is virulent to both *R* genes. Interestingly, *AvrPiz-t* and *AvrPi2* were mapped in different genomic locations. *AvrPiz-t* was mapped on chromosome 7 whereas *AvrPi2* was delimited in an unmapped genomic region (G.D. Lu et al. personal communication). Therefore, *AvrPi2*, *AvrPiz-t*, and *AvrPiz* are most likely located in three different genomic locations. The *AvrPiz-t* was further mapped within a BAC clone 7BG7. Complementation test with all the candidate genes revealed that a predicted gene 7BG7.15 was *AvrPiz-t*. Interestingly, it encodes a small secreted protein. The *AvrPi2* was also delimited within a BAC clone and further identification of the candidate genes is underway.

7 Future Perspectives

The fact that multiple resistance genes controlling broad but different resistance specificity to *M. oryzae* makes the *Pi2/9* locus a quite unique genetic locus to understand the molecular mechanism underlying the broad spectrum resistance to rice blast. Cloning of more alleles, e.g., *Pigm(t)* and *Pi40(t)* will facilitate the determination of the sequence region controlling the resistance specificities. To develop the durable resistance of elite rice cultivars, it will be more applicable to deploy

the broad-spectrum resistance genes than the ones with narrow-spectrum in rice breeding program. The multiple resistance genes located in the different genomic location at the *Pi2/9* locus (e.g., 23 kb apart between *Pi2* and *Pi9*) make it feasible to further pyramid them into one cultivar, which should make the resistance at the *Pi2/9* locus more durable. It will be another strategy to pyramid the resistance at the *Pi2/9* locus with the ones in other loci in rice breeding program.

Isolation of the cognate *Avr* genes to the resistance genes at the *Pi2/9* locus makes it possible to analyze the mechanism of their co-evolution at the molecular level. Sequence variation and dynamics of *Avr* genes in natural *M. oryzae* populations should be surveyed, which would provide important clues to understand the broad-spectrum resistance mediated by the *R* genes at the *Pi2/9* locus. The analysis of the interaction between R and Avr proteins will also be carried out to reveal the molecular mechanism underlying the different recognition ability in different *R/Avr* gene pairs.

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Understanding the Co-evolution of the Rice Blast Resistance Gene *PI-TA* and *Magnaporthe oryzae* Avirulence Gene *AVR-PITA*

Yulin Jia, Xueyan Wang, Stefano Costanzo and Seonghee Lee

Abstract The *Pi-ta* gene in rice effectively prevents infection by races of *Magnaporthe oryzae* that contain the corresponding *AVR* gene, *AVR-Pita*. *Pi-ta* is a putative cytoplasmic protein with a centrally located nucleotide binding sites (NBS) and a leucine rich domain (LRD) at the carboxyl terminus. The *Pi-ta* gene has been deployed effectively in preventing rice blast in the southern US since 1990. *AVR-Pita* encodes a predicted metalloprotease, and its processed form, $AVR\text{-}Pita_{176}$, was shown to directly bind with the Pi-ta protein in triggering effective defense responses. Variants of *AVR-Pita* were identified in many contemporary *M. oryzae* races and in isolates collected during the last 30 years in the US. Sequence analysis of these *AVR-Pita* variants revealed that the *AVR-Pita* protein might be under diversified selection. Most recently, sequence analysis of the *Pi-ta* variants in six *Oryza* species (*O. sativa*, *O. glaberrima*, *O. officialis*, *O. rufipogon*, *O. barthii* and *O. nivara*) revealed that functional nucleotide polymorphism at the position of 918 (FNP918) is present among all these *Oryzae* species, and *Pi-ta* may be under balanced selection. Our results suggest that *Pi-ta* co-evolves with *AVR-Pita*, and that rice engages trench warfare with *M. oryzae* during the host and pathogen co-evolution.

Keywords *Pi-ta* · *AVR-Pita* · Co-evolution · Balanced selection

1 Introduction

Blast disease of rice caused by the filamentous ascomycete *Magnaporthe oryzae* (*Magnaporthe grisea* [T.T. Hebert) M.E. Barr] is a continuous threat to rice production and global food supply (Lee 1994). *M. oryzae* is also the causal agent for a number of other cereal diseases including wheat blast disease. The fungus can infect plants through leaves as well as the roots and is well known for its rapid

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adaptability (Kawasaki 2004). Resistance to *M. oryzae* in rice follows a gene-for-gene specificity where major resistance *R* genes (*Pi* for *Pyricularia*) are effective in controlling infection by races of *M. oryzae* possessing corresponding avirulence (*AVR*) genes (Flor 1971; Silue et al. 1992; Jia et al. 2000). Until now, at least 40 major *Pi* genes have been described in different rice germplasm worldwide, and six of them have been cloned (McCouch et al. 1988; 1994; Yu et al. 1996; Wang et al. 1999; Bryan et al. 2000; Gowda et al. 2003; Qu et al. 2006; Chen et al. 2006; Zhou et al. 2006; Liu et al. 2007). In the pathogen, 25 *AVR* genes have been described and five of them, *PWL1*, *PWL2*, *AVR1-CO39*, *AVR-Pita (AVR2-YAMO)*, and *ACE1* were molecularly characterized (Kang et al. 1995; Sweigard et al. 1995; Farman and Leong 1998, Dioh et al. 2000; Farman et al. 2002; Orbach et al. 2000; Böhnert et al. 2004). The *AVR* genes in *M. oryzae* are known to be highly unstable; in fact, rapid occurrences of the virulent isolates (race shift isolates) have already challenged the effectiveness of *R* gene-mediated resistance (Valent 1997; Correll et al. 2000).

2 The *PI-TA* Locus

The *Pi-ta* gene, one of the major *R* genes in Katy, was found responsible for defeating the major races of *M. oryzae* in the US (Moldenhauer et al. 1990; Moldenhauer et al., 1992; Jia et al. 2004b). *Pi-ta*, a single copy gene, is located at 10.6 Mb near the centromere of chromosome 12, a region that often associates with recombination suppression (Bryan et al. 2000; Chen et al. 2002). *Pi-ta* encoded by two open reading frame (ORF) sequences interrupted by a single intron, is predicted to be a cytoplasmic protein with 928 amino acids, a centrally located nucleotide-binding site, and an imperfect LRD at the carboxyl terminus. A single amino acid alanine at position 918 of the Pi-ta protein determines its resistance specificity (Bryan et al. 2000; Jia et al. 2000).

We recently sequenced *Pi-ta* variants in 52 accessions of six *Oryza* species (*O. sativa*, *O. glaberrima*, *O. officialis*, *O. rufipogon*, *O. bartthii*, and *O. nivara*) in the search for additional resistant *Pi-ta* proteins (X.Wang, Y. Jia, et al., unpublished). Until now, sixteen *Pi-ta* variants were predicted from these DNA sequences. The sequence variations were mainly identified in the intron region, which will be removed before translation and has no affect on the final protein. The ratio of nonsynonymous substitution (*Ka*) to synonymous substitution (*Ks*) is less than 1 for *Pi-ta*. Nine proteins similar to the *Pi-ta* protein were predicted from the coding regions of the *Pi-ta* variants. All of these 9 *Pi-ta* variants were transcribed constitutively as confirmed by RT-PCR experiments. Computational alignment of all putative protein sequences and artificial infection assays reveal that the presence of alanine at position 918 correlates with the resistant interaction, whereas, the presence of serine in the same position correlates with susceptibility. Thus far, only one *Pi-ta* allele was identified conferring resistance to races of *M. oryzae* containing *AVR-Pita*, while all other remaining variants were predicted to be susceptible *pi-ta* alleles. These data suggest that the FNP918 of the *PI-TA* protein is shared among all *Oryza* species examined.

The majority of rice accessions containing these 9 *Pi-ta* variants (with serine) were susceptible to races of *M. oryzae* containing *AVR-Pita* except one group. For example, Raminad strain #3, a rice cultivar from India in this group is also resistant to races of *M. oryzae* lacking *AVR-Pita*. One possibility is that the *Pi-ta* variant in Raminad strain #3 (RNS3) possesses a novel *R* gene that is recognized by several races of *M. oryzae*. To prove the presence of a novel *R* gene in RNS3, an F_2 population consisting of the cross of RNS3 with a susceptible experimental line RU9101001 is being analyzed using a dominant marker identified from the *Pi-ta* variant from RNS3.

The DNA sequences of the intron region and the coding region spanning FNP918 were analyzed and a correlation was established between rice accessions containing the resistant *Pi-ta* allele, as identified by the *SNLP* marker (Jia et al. 2004a), and a new FNP was discovered at the nucleotide position 4234 of the intron region. The nucleotide G at this position correlates with resistance whereas the nucleotide A at this position associates with the susceptibility (X. Wang and Y. Jia, unpublished data). To date, these highly conserved nucleotide sequences in rice accessions enabled us to develop user-friendly DNA markers to tag the *Pi-ta* gene alleles by marker-assisted selection (Jia et al. 2002; 2004a,b; Jia 2003; Johnson et al. 2005).

To survey the presences of the *Pi-ta* allele, a selection consisting of 1,790 rice entries from 114 countries and representing 70% of the total genetic diversity present in all the accessions from the rice world collection of the USDA (Yan et al., 2007), was used to screen for the *Pi-ta* gene using a marker derived from an *InDel* and an SNP from the *Pi-ta* gene (Jia et al. 2004a; Fjellstrom et al., unpublished data). These *Pi-ta* containing accessions were then inoculated with a race of *M. oryzae* containing *AVR-Pita* to examine their disease reactions. Our data suggest that the *Pi-ta* gene is distributed in 142 rice germplasm including the Chinese indica cultivars, Teqing and Guichao 2 (X. Wang and Y. Jia et al. unpublished data).

3 The *PTR(T)* Locus is Required for *PI-TA/PI-TA*^{2}

Seeds of the cultivar Katy (*Pi-ta/Pi-ta*, $Pi\text{-}ta^2/Pi\text{-}ta^2$, $Pi\text{-}K^s/Pi\text{-}K^s$) were mutagenized with 26.3 Gy fast neutron (FN) irradiation at the Oak Ridge Laboratory, Tennessee, US (Bryan et al. 2000; Rybka et al. 1997). A total of 5,781 M_1 panicles were recovered, and 7–10 seeds of each M_2 family from individual M_1 plants were screened for blast disease susceptibility in the greenhouse using a race of *M. oryzae* containing *AVR-Pita*. A new locus, *Ptr(t)* referencing *Pi-ta* required [(t = temporary)] was identified from the rice cultivar Katy containing *Pi-ta*, $Pi\text{-}ta^2$ and $Pi\text{-}k^s$. The mutation at the *Ptr(t)* locus in mutant 2,354 completely suppress the resistance mediated by both *Pi-ta and* $Pi\text{-}ta^2$, however, the resistance mediated by $Pi\text{-}k^s$ remains unaltered (Jia et al., 2005; Jia and Martin, 2008). This result suggests that mutations did not disrupt an essential component in the downstream signaling pathways, and *Ptr(t)* is more likely involved in the *Pi-ta*-mediated defense responses. Pathogenicity assays using races of *M. oryzae*

[(O-137 (*AVR-Pita*, *avr-pita*^{2}) CP3337 (*avr-pita*, *avr-pita*^{2}.), *Pi-ta* O-135 (*avr-pita*, *AVR-Pita*^{2}) CP753 (*avr-pita*, *avr-pita*^{2})] recognizing both *Pi-ta* and *Pi-ta*^{2} indicate that both *Pi-ta* and *Pi-ta*^{2}-mediated resistance was abolished in the mutant 2354 (B. Valent and Y. Jia, unpublished data). A series of *Pi-ta/Ptr(t)*, *Pi-ta/ptr(t)* and *pi-ta/ptr(t)* homozygotes were crossed, and their progeny were analyzed to determine the *Ptr(t)* map location. Results of the segregation and allelic tests suggest that the *Ptr(t)* gene co-segregates with both *Pi-ta* and *Pi-ta*^{2}, and no recombinants were identified in these crosses (Y. Jia and R. Martin, unpublished data). A recombinant inbred line population consisting of 236 individuals of the cross of RU9101001 with Katy was used to identify recombinants between *Pi-ta* and *Ptr(t)*. In all cases, resistance to races of *M. oryzae* containing *AVR-Pita* co-segregates with the *Pi-ta* gene, and no recombinant between *Pi-ta* and *Ptr(t)* was identified from this population. The population size may be too small to detect the recombination event between *Pi-ta* and *Ptr(t)*. It is also possible that the observed lack of recombination at the *Pi-ta/Ptr(t)* region could be due to recombination suppression at the centromeric region (Y. Jia and R. Martin unpublished data; Chen et al. 2002). Currently, an F_2 population of 4,000 individuals of the cross of *Pi-taPtr(t)* and *Pi-taptr(t)* homozygotes is being analyzed to identify recombinants (S. Costanzo and Y. Jia, unpublished data). Cloning and characterization of *Ptr(t)* should reveal the roles of *Ptr(t)* in *Pi-ta*-mediated signal recognition and transduction.

4 The *AVR-PITA* Locus

The *AVR-Pita* gene of *M. oryzae* determining the efficacy of the *Pi-ta* gene is located at a telomeric region of chromosome 3 (Orbach et al. 2000). *AVR-Pita* encodes a predicted metalloprotease with a conserved zinc protease motif that is predicted to be important for its function as an *AVR* gene (Jia et al. 2000; Orbach et al. 2000). The existence of the *AVR-Pita* variants was recently examined in races of *M. oryzae* as well as isolates collected over the 30 year period 1970–2000 from the southern US by Dr. Tony Marchetti (Rice Research Unit, Beaumont, Texas). A pair of *AVR-Pita* specific primers was developed to determine the existence of *AVR-Pita* in isolates of *M. oryzae* (Zhou et al. 2007). A total of 160 isolates were tested, and resulting PCR products were cloned into plasmids for sequencing. Variations of the DNA sequence were mainly identified in the coding region (Y. Dai and Y. Jia, unpublished data). Only minor variations of DNA sequences were identified among the *AVR-Pita* variants of the avirulent isolates/races. Thus far, the ratio of nonsynonymous substitution (*Ka*) to synonymous substitution (*Ks*) is greater than 1 for *AVR-Pita*.

In virulent and race shift isolates, large sequence variations in *AVR-Pita* were detected by Southern blot, and by PCR analysis using *AVR-Pita* specific primers (Zhou et al. 2007). Deletions, insertions, point mutations leading to the frame-shift were observed among the *avr-pita* variants in virulent races of *M. oryzae* (Orbach et al. 2000; Zhou et al. 2007). Variation of DNA sequences nearby the telomere is not unexpected because *AVR-Pita* is located proximately to the telomere of a *M. oryzae*

chromosome (Orbach et al. 2000). Most races of *M. oryzae* regaining their ability to infect rice cultivars containing the *Pi-ta* genes have lost the function of the *AVR-Pita* gene (Lee et al. 2005). The *AVR-Pita* allele was analyzed in a single virulent isolate, B2, collected from a commercial rice field where severe blast disease occurred and resulted in significant economic loss (Lee et al. 2005). The sequence of a complete transposable element Pot3 was identified disrupting the protease motif of the *AVR-Pita* allele in B2 (Zhou et al. 2007). A Pot3 element was found at the promoter region of an *AVR-Pita* allele in one of the virulent isolates recovered in the laboratory (Kang et al. 2001). Based on these findings, we suggest that the fungus may use Pot3 to disrupt the expression of the *AVR-Pita* gene to "bypass" the resistance provided by the *Pi-ta* gene in rice fields.

Investigating *AVR-Pita* interacting genes should shed light on the nature of the substrate for *AVR-Pita* during the invasive growth of the pathogen. The recombinant $AVR\text{-}Pita_{176}$ protein was in-frame fused to GAL4 DNA binding domain as bait to screen a two hybrid cDNA library using mRNA from leaves after inoculation with an incompatible *M. oryzae* pathogen in the yeast two-hybrid system. One of the *AVR-Pita* interacting proteins, *AVI3* was identified and further analyzed (Y. Jia and B. Valent, unpublished data). *AVI3* is a single copy gene on chromosome 1 encoding a putative transcription factor. *AVI3* was transiently induced with the fungal elicitors in rice suspension cells. Interestingly, *AVI3* was also induced by *Rhizoctonia solani*, the causal agent of the rice sheath blight disease. The role of *AVI3* in defense responses is being currently investigated.

5 *PI-TA* Interacts with *AVR-PITA*

Until now, only one *Pi-ta* allele confers resistance and nine *pi-ta* alleles conferring susceptibility have been identified from the USDA rice germplasm core collection. These *pi-ta* variants may encode the proteins to recognize the products of the *AVR* genes in different races of *M. oryzae* or the products of the *AVR* genes from different pathogens. The *PI-TA* protein differs from the pi-ta protein by a single amino acid, due to the substitution of a single nucleotide (Bryan et al. 2000). Frequent occurrences of nucleotide substitutions in the non-coding region are indicative for a gene that is predicted to be under balanced selection. Whether or not *Pi-ta* is under balanced selection needs to be further investigated with larger sampling in *Oryza* species. It is also possible that the *pi-ta* variant is modified to recognize the diverse forms of the products of the *AVR-Pita* variants. The finding that the *Ptr(t)* locus is required for both *Pi-ta* and $Pi\text{-}ta^2$ mediated resistance raising a possibility that *Ptr(t)* is also involved in modifying *AVR-Pita*. Results from our limited sampling and sequence efforts already showed that the majorities of the *AVR-Pita* variants in avirulent isolates encode highly similar proteins with minor alterations of amino acids. The conserved functional domain, i.e., the protease motifs of these predicted proteins were found intact. The *AVR-Pita* protein may be an active protease that plays an important role in invasive growth of the pathogen. Regardless of whether

AVR-Pita is meant to promote the disease, recognition capacity of *Pi-ta* of the host to *AVR-Pita* of the pathogen suggests a trench warfare relationship occurs between a slowly evolved host protein Pi-ta and a fast-evolved pathogen protein AVR-Pita.

6 The Stability of *PI-TA*-Mediated Resistance

The *Pi-ta* gene is one of the most effective *R* genes deployed for blast resistance worldwide. In the southern US, *Pi-ta* has been effectively deployed to control rice blast disease since 1990 (Moldenhauer et al. 1990). The effectiveness of the *Pi-ta* gene in the US could be due to its recognition specificity to a protease that may play important roles in pathogenesis and fitness in rice production areas. It is equally possible that environmental conditions favoring *M. oryzae* epidemics have been marginal, thus, virulent races such as IE-1k that overcome the resistance provided by *Pi-ta* were unable to cause noticeable economic loss except for occasional severe hits on small acreages (Lee et al. 2005). Similarly, the deployment of rice cultivars with broader genetic backgrounds and integrated cultural practices probably account for the effectiveness of the blast resistance provided by *Pi-ta*. A uniform genetic background encourages the adaptation of the pathogen. The recent rapid deployment of hybrid rice worldwide will present additional challenges due to an apparent limited availability of germplasm suitable for use as parents in seed production.

7 The Downstream Defense Pathways

Rapid expression of defense genes after the infection of *M. oryzae* is one common feature of the resistance reaction in host-pathogen interactions. Under heavy artificial inoculation, rapid accumulation (within 24 h) of transcripts of the defense genes, PR-1, ß-glucanase, and chitinase in plants containing the *Pi-ta* gene, suggesting that plants respond to the pathogen infection quickly. These defense genes could be used as RNA markers to detect early resistance response to blast (Wang et al. 2007b). Conservation of defense gene activation by *R* genes across the plant kingdom led to speculation that resistance pathways to different pathogens may be conserved. The homologous DNA sequence of the *Pi-ta* gene also exists in other cereals including barley, maize, oat, rye, wheat, and also crabgrass, foxtail millet and weeping lovegrass based on hybridization under low stringency conditions (Bryan et al. 2000). Highly homologous sequences of the *Pi-ta* gene have only been found, thus far, in maize and rye (http://www.ncbi.nlm.nih.gov). Characterization of structural and functional properties of these *Pi-ta* homologs in additional crops should help to understand whether or not these *Pi-ta* homologs are also *R* genes. For example, rice that is immune to all rust fungi should be examined to see whether *Pi-ta* plays a role in preventing the rust disease. Efforts to determine if the *Pi-ta* gene

is useful in preventing soybean rust is being undertaken by Dr. Henry T. Nguyen at the National Center for Soybean Biotechnology, University of Missouri-Columbia, Missouri, USA.

8 A Working Model of *PI-TA*-Mediated Defense Responses

The recombinant AVR-Pita protein infiltrated outside the plant cells, and in the intercellular spaces was unable to induce *Pi-ta*-mediated defense responses (B. Valent, unpublished data). The removal of 47 amino acids at the amino terminus of the AVR-Pita protein created a putative processed AVR-Pita$_{176}$. AVR-Pita$_{176}$ expressed inside the plant cell with activation of *Pi-ta*-mediated resistance led to a hypothesis that the product of *AVR-Pita* acts inside plant cells. Direct interaction observed between LRD of the Pi-ta protein and AVR-Pita$_{176}$ suggests that Pi-ta recognizes AVR-Pita$_{176}$ directly in triggering resistance (Jia et al. 2000). Research is presently in progress in four new areas: (1) the proteins that are involved in processing the AVR-Pita protein; (2) the molecular mechanisms of the translocation of the AVR-Pita protein into the plant cell; (3) whether the binding of Pi-ta to AVR-Pita$_{176}$ requires additional plant proteins, such as Ptr(t), a protein that is required for both the functions of *Pi-ta* and *Pi-ta*^{2} (S. Costanzo and Y. Jia, unpublished data) and 4) the cellular localization of the Pi-ta and AVR-Pita$_{176}$ proteins (Fig. 1).

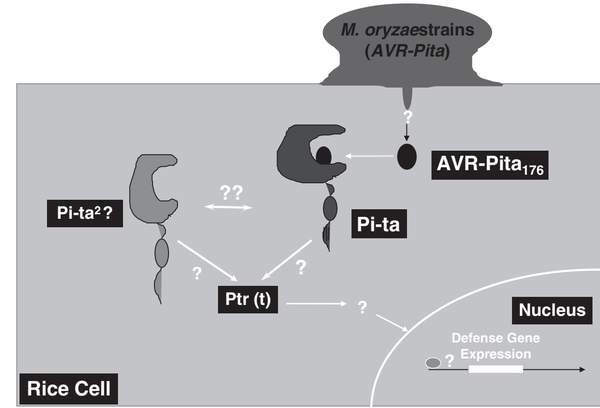

Fig. 1 A working model of *Pi-ta* mediated defense response. The AVR-Pita protein is processed to be AVR-Pita$_{176}$ when AVR-Pita$_{176}$ is present inside plant cell it binds to the Pi-ta protein, and interacts with Ptr(t) either directly or indirectly in activating and transducing *Pi-ta* mediated defense responses
Note: the action site of the *Pi-ta*^{2} gene is unknown

9 Future Perspectives

Rice produced in the US plays an important role in the international market. The majority of rice in the US is currently being grown in the south. The deployment of *R* genes combined with integrated cultural practices is an important component

of sustainable rice production. In the southern US, since severe blast disease epidemics occurred during the 1980s, *Pi-ta, Pi-ta*^{2} and *Ptr(t)* was bred into several elite cultivars. Katy (Moldenhauer et al., 1990) released in 1990 is known to originally derived from a landrace indica variety Tetep from Vietnam (Bryan et al. 2000; Jia et al. 2003). Subsequently, Katy was used as the *Pi-ta* donor for the elite US cultivars, Madison (McClung et al. 1999), Drew (Moldenhauer et al. 1998); Kaybonnet (Gravois et al. 1995), Cybonnet (Gibbons et al. 2006); Spring, and Ahrent (Moldenhauer et al. 2007a,b). The *Pi-ta* gene in Banks was derived from a mutant of irradiated Bonnet 73 (Moldenhauer et al., 2007c). In Japan, the cultivar K1 was identified to be the original donor for *Pi-ta* present in Yashiro-mochi (Kiyosawa 1966) (40 years). The presence of the *Pi-ta* gene has been recently confirmed in IR64, the most widely grown rice cultivar in the world (Wang et al. 2007a).

In the future, real challenges lie ahead in identifying the *R* genes that provide overlapping resistance spectra to a wide range of races of the pathogen. The bottleneck, however, is the uncertain quantity of *R* genes present in any rice germplasm. Similarly, it is difficult to determine how many *AVR* genes are present in an isolate of the pathogen. Quarantine restrictions often preventing the exchange of blast isolates makes the confirmation of the identity of *R* genes difficult, thus, the confirmation of *R* genes currently relies on the limited exchange of rice germplasm. An *R* gene in a rice germplasm can be identified using molecular markers. Increasing efforts on cloning additional *Pi* genes should lead to the development of more "perfect markers" to allow more reliable identification of these genes (Jia 2003; Jia et al. 2002; Jia et al. 2004a,b; Fjellstrom et al. 2004).

We are continuing with studies of the roles of *AVR-Pita* in fitness and pathogenicity. Resulting knowledge not only will provide insights into molecular mechanisms of evolution of the *AVR-Pita* gene of *M. oryzae*, but also useful in predicting the stability of *Pi-ta* based blast resistance in rice cultivars. The almost routine new occurrences of virulent strains of *M. oryzae* demand identification and pyramiding of additional blast *R* genes to achieve durable blast resistance. In the US, two novel genes *Pi-41* (t) and *Pi-42* (t) were recently identified from Zhe733, an indica cultivar from China that control a virulent race IE1k (Wamishe et al. 2005; S. Lee, Y. Wamishe and Y. Jia, unpublished data). Isolation of the corresponding *AVR* genes to *Pi-41* (t) and *Pi-42* (t) in *M. oryzae* should provide more valuable resources to understand the pathogen and host interaction in the rice blast system. In the long term, these new pairs of *R/AVR* genes should help to elucidate the molecular basis of *R* gene-mediated signal recognition and transduction, and help to understand the co-evolution of *R* genes in the host and *AVR* genes in the pathogen.

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Genetic and Molecular Analyses of Blast Resistance in a Universal Blast Resistant Variety, Digu

Xuewei Chen, Junjun Shang, Cailin Lei, Jichen Xu, Shigui Li and Lihuang Zhu

Abstract Digu, an *indica* rice variety, is an important genetic resource for its universal resistance to rice blast disease in China, from which, three new resistant (R) genes, *Pi-d(t)1*, *Pi-d2*, and *Pi-d3*, have been identified by genetic and molecular assays. Among them, the broad spectrum *R* genes, *Pi-d2* and *Pi-d3* have been cloned. *Pi-d2* encodes a B-lectin receptor-like kinase while *Pi-d3* an NBS-LRR protein. As *Pi-d2* and *Pi-d3* belong to different classes of *R* genes, they may confer resistance to the pathogen *Magnaporthe grisea* through different signaling pathways. Thus we conclude that the universal resistance of rice Digu is probably due to its complicated resistance mechanisms mediated by different signaling cascades.

Keywords Resistant gene · Rice blast · Rice (*Oryza sativa* L.)

1 Introduction

Rice blast, caused by the pathogen *Magnaporthe grisea*, is one of the most destructive factors in rice production. Developing blast-resistant varieties is the most economical and effective method of controlling this disease. However, most newly bred varieties with simple resistance to blast may quickly lose their resistance due to the rapid variation of the blast pathogens. Therefore, a preferable way is to use the rice resources with broad-spectrum resistance (R) genes to develop rice varieties in breeding. So far, more than 40 blast R genes have been identified in rice resources with broad-spectrum (Chen et al. 2004; Wu et al. 2007). Among them, some were identified from broad-spectrum resistant rice, such as three R genes and 10 QTLs from Moroberekan, a rice variety with durable resistance to blast disease (Ann, 1994; Chen et al. 1999; Wang et al. 1994), at least four genes from Tetep (Wang et al. 1994), two from LAC23 (Mackill and Bonman 1992), three from

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Pai-Kan-Dao (Ann 1994), two from Gumei 2(Wu et al. 2005), three R genes and five QTLs from San-Huang-Zan 2(Wu et al. 2004), *Pi2* from indica rice variety 5173(Zhou et al. 2006), *Pi9* from *Oryza minuta*(Qu et al. 2006), *Pi33* from IR64(Berruyer et al. 2003), *Piz* from Zenith (Conaway-Bormans et al. 2003; Hayashi et al. 2006), and Piz-t from TKM1 (Zhou et al. 2006). Among them, *Pi2*, *Pi9*, and *Piz-t* were cloned and identified as broad-spectrum R genes (Qu et al. 2006; Zhou et al. 2006).

Digu, a Chinese *Indica* (*O. sativa subsp. Indica*) cultivar, was bred from a local *indica* cultivar, Gunong 13, showing universal resistance to blast in the south of China, and is one of the most important genetic resources for rice blast resistance breeding in China. It was found that none of 156 blast isolates collected from China and Japan could cause disease on Digu (data not shown). We reported here that three new R genes, *Pi-d(t)1*, *Pi-d2*, and *Pi-d3*, were identified from this cultivar, and two of them were cloned. The molecular structures of the R proteins encoded by the two cloned genes, *Pi-d2*, and *Pi-d3*, imply that different molecular mechanisms are involved in Digu's resistance to rice blast. Our studies on the two R genes demonstrated that the universal resistance of Digu is probably due to the complicated molecular mechanisms involved in its broad-spectrum blast resistance.

2 Materials and Methods

For blast strains, inoculum preparation, inoculation and disease evaluation, see the reference of (Chen et al. 2004). For the molecular markers, gene mapping, gene cloning, rice stable transformation, and protein structure assay, see the references of (Chen et al. 2004; 2006). The main websites used in this study are http://rgp.dna.affrc.go.jp/E/index.html; http://www.tigr.org/; http://www.ncbi.nlm.nih.gov/; http://smart.embl-heidelberg.de/; and http://www.expasy.ch/prosite/.

3 Results

3.1 Genetic Analyses and Molecular Identification of Three New Blast R Genes in Digu

In order to explore the genetic rule of the blast resistance of Digu, we crossed Digu with Li Jiang Xin Tuan Hei Gu (LTH) and TP309, respectively; the former is an universal blast susceptible variety (Ling et al. 2000; Tsunematsu et al. 2000) while the latter is known as susceptible to most blast isolates and easy to be transformed by *Agrobacterium tumefaciens* (Barakat et al. 2000). The F2 population developed from the cross of Digu X LTH or Digu X TP309, were inoculated with three most compatible strains, ZB13 and ZB15 from South China and zhong-10-8-14 from North China. The resistance of Digu to ZB13, ZB15 or Zhong-10-8-14 was segregated in the two populations; the segregation ratio in either population upon ZB13

Table 1 The R/S segregation in the F2 progenies from the crosses of DG/LTH and DG/TP309

Strain name	Progenies	Total number of plants	Number of R/S plants		Chi-Square test		
			R	S	Expected ratio(R:S)	χ^2	P0.05,0.01
ZB13	(DG/LTH)F2	270	212	58	3:1	1.782	3.84–6.63
ZB15	(DG/LTH)F2	472	372	100	3:1	3.406	3.84–6.63
Zhong-10-8-14	(DG/TP309)F2	473	449	24	15:1	0.04	3.84–6.63

χ^2 = the actual value of Chi-square test for the resistance/susceptibility ratio; P = the expected value of Chi-square test for the resistance/susceptibility ratios with the probabilities of 95–99%: R = resistant; S = susceptible.

and ZB15, respectively, agreed with 3:1 but the ratio in either population upon Zhong-10-8-14 agrees with 15:1 (Table 1). These results suggest that the respective resistance of Digu to ZB13 and ZB15 is controlled independently by a single dominant gene, and the resistance to Zhong-10-8-14 is controlled by two dominant genes. Since there had been no resistance gene identified in Digu, based on the segregation data we assumed that there exist at least three blast R genes, named *Pi-d(t)1*, *Pi-d2*, and *Pi-d3*, respectively. To determinate if these genes are same to some known blast R genes, a set of rice varieties carrying the identified genes and the progenies generated from their respective crosses with LTH were challenged by ZB13, ZB15, and Zhong-10-8-14, respectively. The results suggested that the *Pi-d(t)1*, *Pi-d2*, and *Pi-d3* are different from all known R genes. The three genes are molecularly mapped on rice chromosomes 2 [*Pi-d(t)1*] and 6 (*Pi-d2*, *Pi-d3*), respectively (Fig. 1).

3.2 Cloning and the Protein Structures of **Pi-d2** *and* **Pi-d3**

The *Pi-d2* and *Pi-d3* were isolated by map-based and *R* gene prediction strategies, respectively. Transgene's complementary test showed that these two cloned genes, *Pi-d2* and *Pi-d3*, have the resistance to ZB15, and Zhong-10-8-14, respectively. The *Pi-d2* gene encodes a B-lectin receptor-like kinase with 825 amino acids (Fig. 2). It is plasma-membrane localized. The C terminus contains a classic Ser-Thr cytoplasmic protein kinase catalytic domain. Within this domain, the consensus sequence GQGGFG (amino acids 513 to 418) is characteristic of an ATP-binding site GXGXXG and the sequence IVHCDIKPENVLL (amino acids 622 to 634) indicates Pi-d2's serine-threonine specificity. The N terminus from residue 1 to 32 encodes a hydrophobic domain which may act as a signal peptide targeting the protein to the plasma-membrane. The putative extra-cellular region contains a classic B-lectin domain with the amino acids 48–165 and a PAN domain with amino acids 337 to 418. The motif MXLXXXGNLVV in the B-lectin domain can generate snowdrop bulbs that might be used for mannose binding (Wasano et al. 2003). The conserved core of three disulphide bridges in the PAN domain can be generated from the region of amino acids 337 to 403. The PAN motif is known to mediate

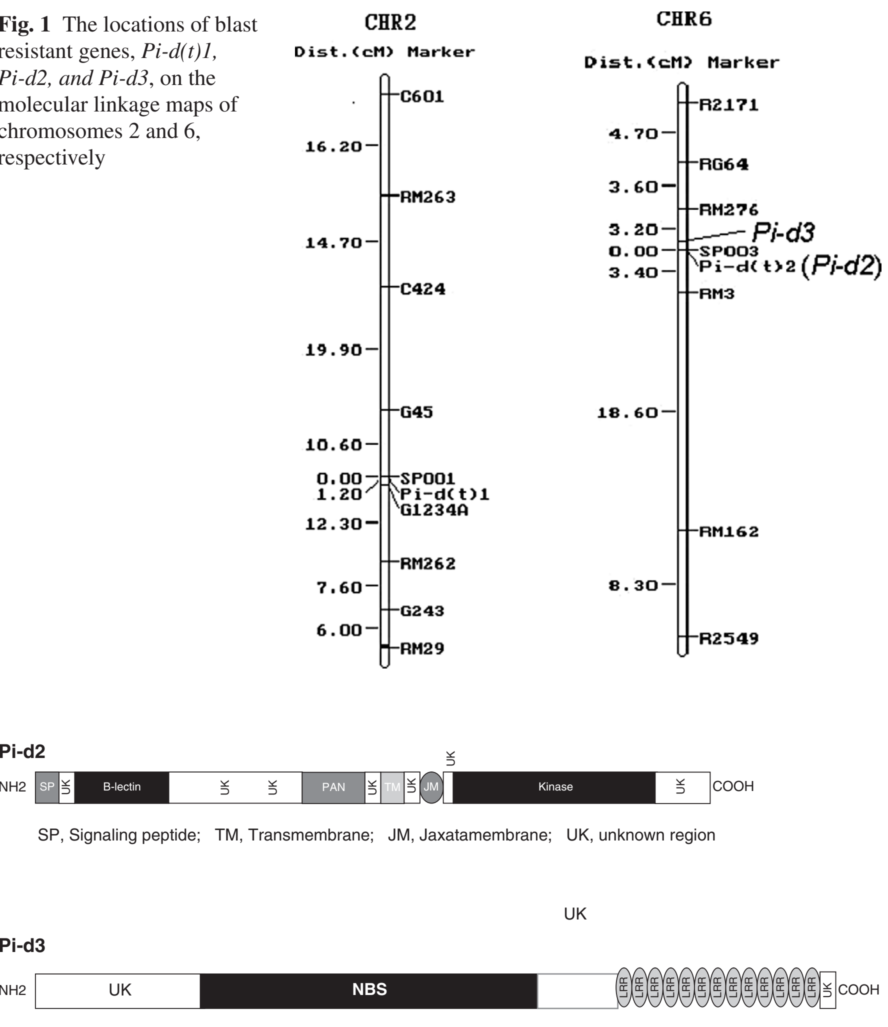

Fig. 1 The locations of blast resistant genes, *Pi-d(t)1, Pi-d2, and Pi-d3*, on the molecular linkage maps of chromosomes 2 and 6, respectively

Fig. 2 The protein structures with conserved domains of *Pi-d2 and Pi-d3*

protein-protein interaction suggesting that Pi-d2 can interact with protein ligands through its PAN motif. Therefore, we presume that either the B-lectin or PAN domains might be recognized by the effector or PAMP from pathogens and make some modifications like phosphorylation, N-glycosylation and N-myristoylation to Pi-d2. The other hydrophobic region (amino acids 436 to 458) that can generate a membrane-spanning helix to function as a transmembrane (TM) region, the ortholog proteins of Pi-d2 in the susceptible rice varieties LTH, TP309, and Nipponbare share the same amino acid change (I $\rightarrow$ M) at position 441 in the TM region. This change

of amino acid may compromise the membrane spanning of Pi-d2 in those susceptible varieties and can not function its disease resistance. Since there is no report about that the B-lectin or PAN domain can function as a signaling receptor in R proteins, the *Pi-d2* gene is a novel R gene.

The R gene *Pi-d3* encodes NBS-LRR protein with a 924-amino acid polypeptide that contains conserved NBS domain from positions 158 to 466 where exist four sequences, GMGGIGKTA (amino acids 202 to 210), KRYVLVLDDVW (amino acids 280 to 290), IGRIILTSRNYDV (Amino acids 307 to 319), and GLPIAI (amino acids 373 to 378), corresponding to kinase 1a (p-loop), kinase 2, kinase 3a, and GLPL motif, respectively. At the C-terminus is the LRR region that comprises 13 imperfect LRR repeats. The MHD motif, MHDILRV (amino acids 502 to 508), and NBS LRR linker motif, EQNFCIVVNHS (amino acids 516 to 526) are present between the NBS domain and the LRR region. At the N-terminus, there is a conserved motif, RSLALSIEDVVD (amino acids 78 to 89), but no TIR or Coiled-coil motif.

We conducted the BLASTP analysis with the protein sequence of Pi-d3. We could find the most similar resistance protein of Pi-d3 in several crops. The protein similarity of Pi-d3 with HV1LRR1 (Genbank accession no: AAD46469) in barley is 40.5%. The protein similarity of Pi-d3 with LRR14 and LRR19 (Genbank accession no: AAK20742 and AAK20736) in wheat are 42.8% and 43.5%, respectively. And the protein similarity of Pi-d3 with RXO1 (Genbank accession no: AAX31149) in maize is 32.5%. The comparison of the ortholog proteins was showed in Fig. 2. The structure and the position of the conserved motif between the proteins are very similar. We also compared the Pi-d3 protein with the isolated rice NBS-LRR R proteins. The similarity between Pi-d3 and Pib, Pita, Pi9, Pi36, and Pi37 protein sequences is 19.0%, 20.5%, 25.4%, 20.6%, and 14.7%, respectively.

3.3 The Resistance Pattern Between **Pi-d2** *and* **Pi-d3** *Transgenic Lines*

The blast R gene *Pi-d2* and *Pi-d3* were both isolated from the *indica* resistant variety Digu and located on the chromosome 6. We compared the resistance of their transgenic lines by using sixteen *M. grisea* strains collected from different Chinese regions. Both *Pi-d2* and *Pi-d3* transgenic lines showed resistance to seven strains among the sixteen strains. But the transgenic lines showed different resistance to six strains (Table 2). The results indicated that *Pi-d2* and *Pi-d3* could be used in resistance breeding program to improve the resistance to rice blast in different regions.

4 Discussion

Digu is an important genetic resource showing resistance to all the inoculated Chinese *M. grisea* strains (data not shown) and has been widely utilized in rice blast resistance breeding in China. In this study, three new resistance genes, *Pi-d(t)1*,

Table 2 The comparison of resistance between TP309, Pid3 transgenic line and Pid2 transgenic line against 16 M. grisea strains

	ZB15	CH43	Sichuan 36	JS2001-108	Sichuan 26	CH706	CH45	CH704	Zhong10-8-14	ZK-10-2	91-65-1	97-3-1	97-27-2	99-20-2	99-26-1	99-26-2
TP309	S	Sh	mS	Sh	S	S	S	S	Sh	Sh	Sh	S	Sh	Sh	S	Sh
Pi-d2 transgenic line	Rh	Rh	mS	Rh	Rh	Rh	Rh	Rh	Sh	Rh	mS	R	S	Rh	mS	Rh
Pi-d3 transgenic line	S	Rh	Rh	Rh	mS	Rh	mS	S	Rh	Rh	mS	Rh	S	Rh	mS	Rh

R = resistant; Rh = highly resistant; mR = moderate resistant; S = susceptible; Sh = highly susceptible; mS = moderate susceptible. At least 9 tillers from 3 plants are used for each treatment.

Pi-d2, and *Pi-d3*, were identified in Digu and mapped on rice chromosomes 2 and 6, respectively. Furthermore *Pi-d2* and *Pi-d3* were successfully cloned. Since these three genes are different from each other and other identified genes, and both of the cloned *Pi-d2* and *Pi-d3*, have broad spectrum resistance to blast strains, Digu may represent another important resistant genetic resource different from others, such as, Moroberekan, Tetep, LAC23, and Pai-Kan-Dao(PKT) (Wu et al. 2007). Its universal resistance may be due to its multi R genes with different spectrums. Both *Pi-d2* and *Pi-d3* were mapped in the region close to the centromere of chromosome 6, where the other four blast disease resistant genes, *Pi2*, *Pi9*, *Piz-t*, and *Piz*, from different varieties had been identified (Wu et al. 2007). Thus we presume that this R gene-rich region may contain more unidentified blast R genes. Considering that most of these genes, such as *Pi-d3*, *Pi2*, *Pi9*, and *Piz*, encode NBS-LRR proteins, there might be some evolutionary relationship among them.

The RLK and NBS-LRR are two main classes of R proteins in planta (Martin et al. 2003, Meyers et al. 2005). In plant, RLK proteins have been greatly expanded being involved in a broad range of cellular processes including growth, development, hormone perception, and plant-microbe interaction (Haffni et al. 2004). The expansion of RLKs and the variations in their extracellular domains reflect the adaptive need to bind a wide diversity of ligands present outside the cell. LecRKs are defined by their extracellular domains which contain the similarity to legume lectins and the lectin domains can bind monosaccharides via hydrogen bonding between conserved amino acids and the oxygens on the hexose sugar (Barrett et al. 2002). However, Pi-d2 may not bind monosaccharides, such as mannose because some of the conserved amino acids required for monosaccharide binding are absent in Pi-d2 (Chen et al. 2006). But, the predicted hydrophobic region in the lectin domain of Pi-d2 may form a structural pocket like in other LecRKs (Barrett et al. 2002; Herve et al. 1999). According to the notion that the hydrophobic pocket has the potential to bind hydrophobic ligands, such as plant hormones or possible pathogen-derived hydrophobic molecules (Barrett et al. 2002; Herve et al. 1999), the Pi-d2 may recognize the blast pathogen through the hydrophobic pocket binding with hydrophobic ligands or possible pathogen-derived hydrophobic molecules. Pi-d2 belongs to the non-RD subclass of kinases because of its lack of the conserved R in subdomain VI like those plant non-RD RLKs involved in disease resistance, such as Xa21(Song et al. 1995), Xa26 (Sun et al. 2004) and FLS2 (Gomez-Gomez and Boller 2000). Interestingly, Dardick and Ronald have found a cross kingdom correlation between the absence of this conserved R and a role in innate immunity in plant, animal and insect (Dardick and Ronald 2006). This would suggest a strong selective pressure, either from pathogens or from biochemical constraints, to adopt and maintain non-RD kinases in activation of innate immune response. The fact that Pi-d2 contains a non-RD kinase domain implies a direct role in pathogen recognition despite the presence of a variable lectin extracellular domain, which has not previously been implicated in defense responses. Upon the recognization of effectors from pathogens, the RLK protein will change its structure to a form, for example, dimerization of FLS2 available to be phosphorylated. The signal is activated along

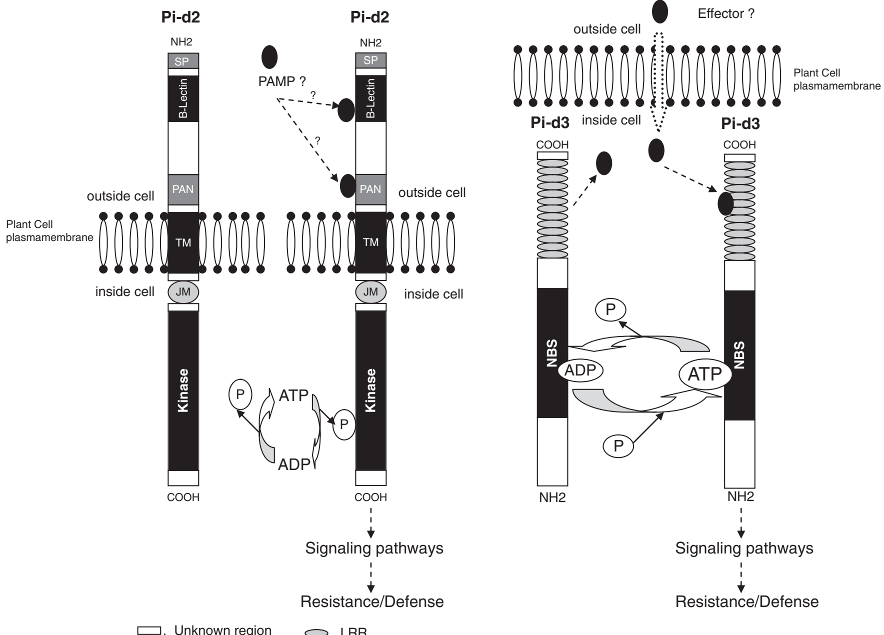

Fig. 3 Proposed models of *Pi-d2 and Pi-d3* in their function to blast resistance. As for *Pi-d2*, upon ligand binding to Lectin or PAN domain, the signal is transduced across the cell membrane domain activating the intracellular kinase domain through phosphorylation, by which, the signal is transduced to defense system. As for *Pi-d3*, The effector from pathogens precipitates from the outside to the inside of plant cell and binds to the LRR of *Pi-d3*. Upon this binding, the signal is transduced to the NBS domain through the binding of ATP with NBS, by which, *Pi-d3* changes its conformation and transduces the signal to the defense system

with RLK proteins' phosphorylation and then transduced to the downstream defense reaction (Fig. 3).

More interestingly, Pi-d2 can interact with some of XA21 binding proteins, such as XB11 (C2 domain-containing protein), XB15 (PP2C), and XB24 (a protein with ATP synthase alpha and beta subunits signature) revealed by the yeast two hybrid screen (data not shown), suggesting that the Pi-d2 mediated blast resistance and the XA21 mediated bacterial resistance might share same pathway(s) in which, XB11, XB15, and XB24 are involved, in their downstream signaling.

The NBS-LRR proteins can detect pathogens through direct or indirect interactions between R proteins and pathogen-derived molecules. For the direct interaction, evidence is derived from the blast resistant protein Pi-ta that can interacts with the effector, Avr-Pi-ta from the rice blast fungus *M. grisea* (Jia et al. 2000), the Flax L protein that can interacts with AvrL from flax rust fungus *Melampsora lini* (Dodds et al. 2006), the *Arabidopsis thaliana* RRS1, a typical TIR-NBS_LRR protein, that can also interact with the bacterial wilt pathogen protein PopP2 in a 'split-ubiquitin' yeast two hybrid experiment (Deslandes et al. 2003). While, a mechanism of indirect detection between NBS-LRR proteins and pathogen effectors is supported by

other evidences, for example, the effectors AvrRpm1 and AvrB, are both detected by RPS2, the R protein from *A. thaliana*, but either of them can not bind RPS2 directly. The tamoto protein Prf indirectly detect AvrPto and AvrPtoB (Mucyn et al. 2006). Since no significant structural characteristics have been found specific for either effector-direct or effector-indirect detection of NBS-LRR proteins, the two possibilities of effector detection exist to the rice NBS-LRR protein, Pi-d3. However, according the model provided by DeYong and Innes (DeYoung and Innes 2006), the signaling is probably activated in a similar way for both modes of direct and indirect detections. In terms of the model, the presence of the pathogen effector alters the structure of the Pi-d3 through direct binding or modification of additional proteins, allowing exchange of ADP for ATP and binding of ATP to the NBS domain which results in activation of signal transduction through the creation of binding sites for downstream signaling molecules and/or the formation of NBS-LRR protein multimers, then the pathogen effector can dissociate from the modified effector targets along with the hydrolysis of ATP and return the NBS-LRR protein to its inactive state (Fig. 3).

Since either of the signaling activation or transduction of NBS-LRR proteins is different from that of RLK R proteins, the Pi-d2 and Pi-d3 mediated disease resistances belong to two types of molecular mechanisms with different signaling cascades. Therefore, the universal resistance of Digu may be due to its at least two different signaling activation systems conferring blast resistance. However, to answer the question that why the Pi-d2 and Pi-d3 with distinguish molecular mechanisms can confer common resistance to some strains is a big but interesting challenge to us in the further work.

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Map-Based Cloning and Breeding Application of a Broad-Spectrum Resistance Gene *Pigm* to Rice Blast

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Abstract Rice blast is one of the most destructive diseases of rice. The identification and utilization of broad-spectrum resistance genes has been the most effective and economical approach to control the disease. A native Chinese variety, GM4, was identified as a broad-spectrum and durable resistant resource. Genetic and mapping analysis indicated that blast resistance to nine isolates of different races in GM4 is controlled by the same dominant locus designated as *Pigm*. The map-based cloning strategy was employed with a large mapping population consisting of 1556 recessive individuals. *Pigm* was finally mapped on chromosome 6. The allelism test showed that *Pigm* was allelic to *Pi-2* and *Pi-9*, two known blast resistance genes. A BAC contig covering the *Pigm* region was constructed and completely sequenced. An NBS-LRR gene cluster encompassing 10 NBS-LRR-type candidate resistance genes was identified in the 120-kb sequenced region, which contains 6 members in the *Pi2* gene cluster. Sequence comparison of the orthologous and paralogous genes in the *Pigm* locus in both resistant and susceptible backgrounds showed that the *Pigm* loci had undergone duplication during the evolution of the resistance gene cluster. Furthermore, our analysis showed that *Pigm* confers broader-spectrum resistance to blast isolates from different rice regions than *Pi9/Pi2/Piz^t/Piz*, indicating its good potential in rice breeding for blast resistance. With molecular markers-assisted selection for *Pigm*, we have succeeded in developing elite hybrid rice lines with broad-spectrum blast resistance.

Keywords Broad-spectrum resistance · *Pigm* · Gene cluster · Resistance breeding

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1 Introduction

Rice blast (*Magnaporthe oryzae*) is one of the most devastating rice disease and causes large yield loss worldwide. *M. oryzae* displays great genetic instability and pathogenic variability, and rapidly breakdowns resistant varieties (Mackill and Bonman 1992). Therefore, rice breeding for blast resistance has been a major breeding target in most of rice growing areas, and the identification and utilization of new resistance (*R*) genes has been the key issue to ensure effective control of the disease. The rice genome contains many *R* genes against blast (Mackill and Bonman 1992; McCouch et al. 1994; Wang et al. 1994; Pan et al. 1996). Molecular cloning of these *R* genes not only provides tools for marker-assisted selection in rice breeding for blast resistance, but also facilitates understanding of the underlying mechanism involved in the *R*-mediated blast resistance. To date, eight of these *R* genes have been isolated by the map-based cloning approach, of which seven, *Pib*, *Pita, Pi2, Pi9*, Piz^t, *Pi36* and *Pi37*, are members of a large *R* gene family encoding receptor proteins containing nucleotide binding site and leucine-rich repeats (NBS-LRR) (Wang et al. 1999; Bryan et al. 2000; Qu et al. 2006; Zhou et al. 2006; Lin et al. 2007; Liu et al. 2007). Another gene, *Pid2*, belongs to a new *R* gene type encoding a receptor-like kinase with extracelluar domain of a bulb-type mannose specific binding lectin (B-lectin) and an intracellular serine-theronine kinase domain (Chen et al. 2006).

However, most of the mapped blast *R* genes confer resistance only to a certain number of blast races. It is well recognized that these genes will certainly provide broad-spectrum resistance when pyramided into the same rice variety. Nevertheless, the identification of *R* genes with broad-spectrum resistance will provide important genetic resources for rice breeding. It has been shown that five *R* genes, *Pi1* (Yu et al. 1991), *Pi2* (Chen et al. 1996, 1999), *Pi3*/*Pi5* (Jeon et al. 2003), *Pi9* (Liu et al. 2002) and *Pi33* (Berruyer et al. 2003), probably confer broad-spectrum resistance. *Pi9* locates on the region containing multiple NBS-LRR resistant gene cluster, and *NBS2-Pi9* is a solo resistance member of the *Pi9* gene cluster, confers broad-spectrum resistance to rice blast. *Pi2*/Piz^t was also mapped on the *R* gene cluster containing *Pi9*, and the functional gene is *NBS4-Pi2* (Zhou et al. 2006). However, the molecular basis of *Pi9/Pi2* in broad-spectrum blast resistance remains to be elucidated (Qu et al. 2006).

In order to identify more useful *R* genes and understand the mechanism of broad-spectrum resistance to rice blast, we conducted genetic analysis and map-cloning of the blast *R* locus *Pigm* conferring broad-spectrum resistance in a Chinese *indica* variety Gumei 4 (GM4), which has been grown in different blast nurseries as a broad-spectrum and durable resistant control. Our previous observation showed that the resistance spectrum of GM4 is broader than those of *Pi1, Pi2, Pi3*, making it a one of good genetic resource in the resistance breeding program (Peng et al. 1996; Shen et al. 2004). In this paper, we reported the results of systematical analysis of the genetics of resistance of GM4 to different blast isolates, and mapping of the *Pigm* locus that contains a gene cluster of 10 NBS-LRR candidate *R* genes on chromosome 6. We also showed that *Pigm* confers broader resistance spectrum

than *Pi2*, *Pi9, Piz*, *Piz^t* and effectively improves resistance in the marker-assistant selection lines.

2 Materials and Methods

2.1 Plant Materials and Blast Inoculation

All the genetic analysis and mapping populations were developed from the crosses between GM4 (*indica*) and three susceptible (S) varieties Cpslo17 (*javanica*), Maratelli (*japonica*) and Suyunuo (*indica*). In addition, the F_2 populations from the crosses between GM4 and 75-1-127 containing *Pi9*, and C101A51 containing *Pi2* were constructed for allelism test. Two-week-old rice seedlings were spray-inoculated with blast spore suspensions (1×10^5 spores/ml) in a dew growth chamber for 24 h in darkness at 26°, and were subsequently kept at 12 h/12 h (day/night), 26° and 90% relative humidity for 6 days. The inject-inoculation method was employed at the tillering stage as described (He and Shen 1989), each tiller of the same individuals was inoculated with one isolate by injecting 0.1ml spore suspensions (2.5×10^4 spores/ml). After 7 days, lesion types on rice leaves were observed and scored 0 (resistant) to 5 (susceptible) according to the standard scale described by Bonman et al. (1986).

2.2 Resistance Spectrum and Allelism Analysis

For allelism test, the F2 populations from the crosses between GM4 and 75-1-127 containing Pi9, and C101A51 containing Pi2 were inoculated with isolate 01-19 to observe resistance segregation. Rice lines GM4(*Pigm*), 75-1-127(*Pi9*), C101A51(*Pi2*), Zenith(*Piz*), Toride1(*Piz^t*), MP3(*Pigm*) were inoculated with 18 blast isolates to differentiate resistance reaction.

2.3 Preliminary and Physical Mapping of Pigm

Six pairs of R and S DNA pools were made from the F_2 populations of the cross of GM4 and Cpslo17 inoculated with 6 different isolates for bulk segregant analysis (BSA) (Michelmore et al. 1991). A total of 120 SSR (simple sequence repeats) markers distributing evenly on 12 chromosomes released by the International Rice Microsatellite Initiative (IRMI) (www.gramene.org/microsat/) were used to construct the R locus linkage map. New CAPS (cleaved amplified polymorphic sequence) and InDels (insertion/deletion polymorphisms) markers for GM4 and Maratelli were designed to further delimit the *R* locus based on SNPs (single nucleotide polymorphisms) and InDels between the *japonica* (Nipponbare) (http://rgp.dna.affrc.go.jp/) and the *indica* (9311) (http://www.genomics.org.cn/)

and GM4 genomes (Xu et al. 2004). Subsequently, a physical map spanning the *R* gene locus was constructed.

2.4 BAC Library Construction and Screen

Isolation of high-molecular-weight (HMW) genomic DNA and the BAC cloning were carried out. Briefly, HMW DNA, isolated from the GM4, was partially digested with the restriction enzyme Hind III. The DNA was then size-fractionated (100–200 kb) using a pulsed-field gel electrophoresis device (CHEF Mapper II, Bio-Rad). DNA purified from low-melting-point agarose was ligated to the Hind III-digested and dephosphorylated BAC vector pIndigoBAC5. The ligation mix was electroporated into *E. coli* DH10B cells. 180 BAC clones were mixed and dissolved in 3.0 ml of liquid LB to form a BAC pool. Half of the cells were stored in a $-80\,^{\circ}\mathrm{C}$ freezer and the other half were used for preparation of BAC plasmid DNA. PCR technique was applied to screen the BAC library using markers co-segerating with *Pigm*. The clone BAC30 was fully sequenced by the shot-gun method.

2.5 Sequence Analysis

We used FGENESH (www.softberry.com) and GENESCAN (http://GENES.mit.edu/GENSCAN.html) for gene prediction from the BAC30 sequence, and further searched against the sequences deposited in GenBank using the BLAST programs (blastn, blastx and blastp, http://www.ncbi.nlm.nih.gov/BLAST/). Pairwise comparisons between genomic sequences of the coding regions of *Pigm* candidate genes were performed using the BLAST program (bl2seq) (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html) and the Matcher program (http://bioweb.pasteur.fr/seqanal/interfaces/matcher.html). The rice repeat database at TIGR (http://www.tigr.org/tdb/e2k1/osa1/blastsearch.shtml) was searched to locate repeats within the sequences and positive hits were classified as either retrotransposons, transposons or miniature inverted repeat transposable elements (MITES).

3 Results

3.1 Pigm *is the Solo Locus Conferring Broad-Spectrum Resistance in GM4*

Inoculation results of the F_1, BC_1F_1, F_2 and BC_1F_2 populations with nine blast isolates incompatible to GM4 were presented in Table 1. The three parents, Suyunuo, Cpslo17 and Maratelli, are highly susceptible to all isolates. Phenotypic segregations of resistance and susceptibility in these F_2 and BC_1F_2 populations fitted the ratio of 3:1, and these in the BC_1F_1 populations followed a 1:1 pattern. Therefore,

Table 1 Genetic and allelism analysis in populations to different isolates (Deng et al. 2006)

Parents and generations	Isolates (races)	Resistant and susceptible individuals		Segregation Ratio	X^2
		R	S		
GM4	CH109 (ZC13)	27	0		
Cplso17		0	30		
F1		26	0		
BC1F1		42	34	1:1	0.84
BC1F2		773	232	3:1	1.89
F2	CH109	282	85	3:1	0.66
	CH174	282	85	3:1	0.66
	CH131 (ZA1)*	282	85	3:1	0.66
GM4	101/1/1(ZA1)	30	0		
Cplso17		0	34		
F2		69	22	3:1	0.05
GM4	101/4/8 (ZA9)	30	0		
Cplso17		0	28		
F2		74	23	3:1	0.05
GM4	CH63 (ZG1)	27	0		
Cplso17		0	9		
F2		86	23	3:1	0.78
GM4	CH199 (ZB1)	25	0		
Cplso17		0	31		
BC1F2		822	259	3:1	0.71
GM4	CH199 (ZB1)	32	0		
Maratelli		0	41		
F1		30	0		
F2		196	61	3:1	0.18
BC1F1		105	97	1:1	0.31
GM4	01-19 (ZB15)	24	0		
Maratelli		0	33		
F2		176	54	3:1	0.28
BC1F1		122	108	1:1	0.85
GM4	CH102 (ZB13)	20	0		
Suyunuo		0	14		
F1		25	0		
F2		147	41	3:1	1.01
BC1F1		55	42	1:1	1.74
GM4	01-19(ZB15)	16	0		
75-1-127 (Pi9)		12	0		
C101A51 (Pi2)		19	0		
F2		717	0		
F2		545	0		

* The same individuals were injection-inoculated with isolates on different tillers.

single dominant genes respectively conferred resistance to nine isolates in GM4, which belonged to the A, B, C and G Chinese race groups (Table 1) (Shen et al. 1998). The further injection inoculation confirmed that a single locus, named *Pigm*, most likely confers broad-spectrum resistance to different races.

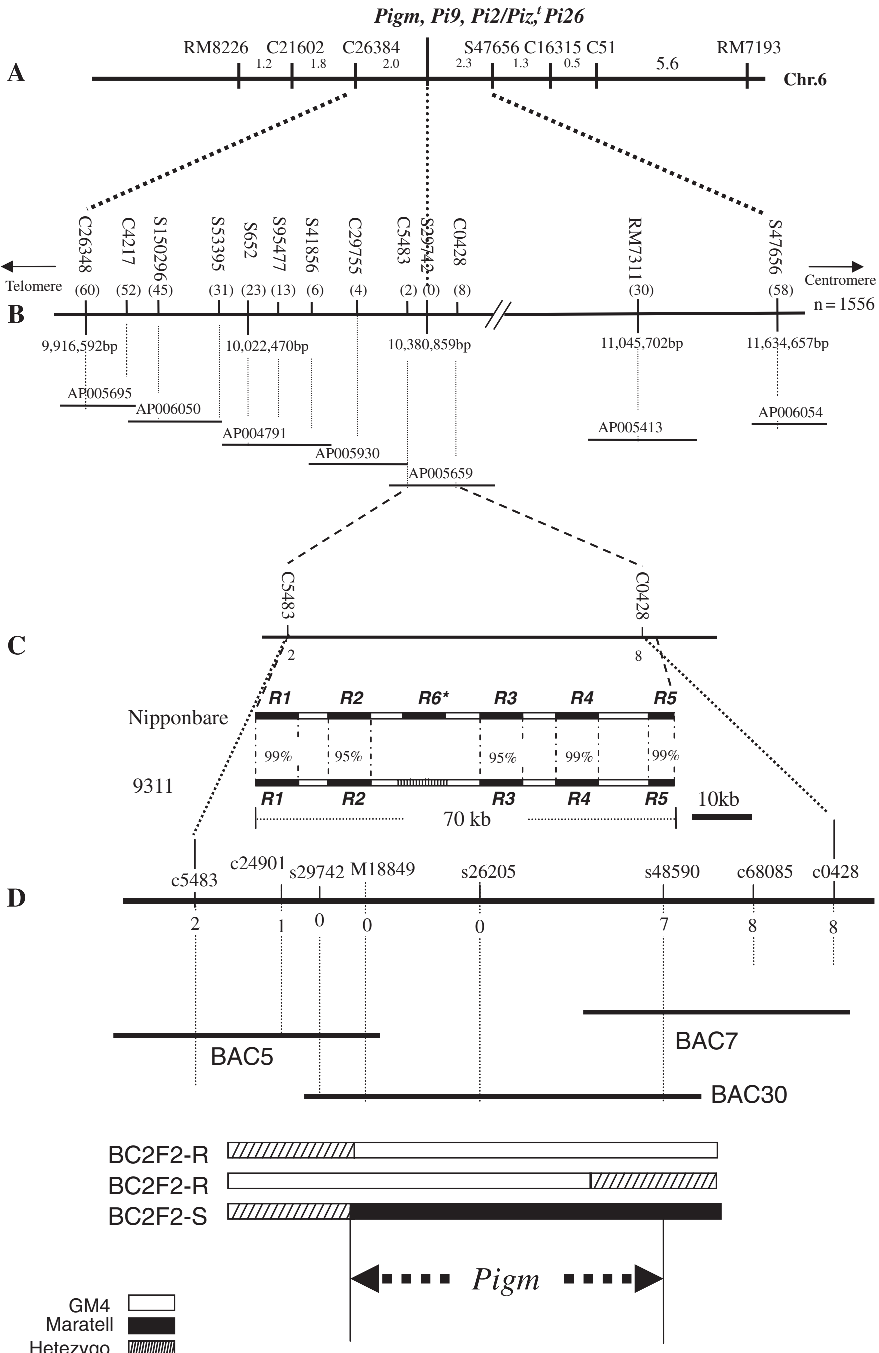

Fig. 1 (continued)

3.2 Physical Mapping of *Pigm*

Through linkage screen with a total of 120 SSR markers distributing with 10–15 cM intervals on 12 chromosomes, *Pigm* was first associated with the marker RM7193 on chromosome 6. The further screening of 306 susceptible/recessive individuals from the cross between GM4 and Cpslo17 revealed a total of 53 recombinant events. Based on this, additional 15 SSR markers were selected in a 40 cM region around RM7193. As a result, *Pigm* was delimited to a 14.7 cM region between two SSR markers, RM7193 and RM8226. To further localize *Pigm* on chromosome 6, we developed 10 CAPS and InDels markers between RM7193 and RM8226 according to sequences of the *japonica* Nipponbare and the *indica* 9311 genomes, and found 4 markers displaying polymorphism between GM4 and Cpslo17. Hence *Pigm* locus was further delimited within a 4.3 cM region between the two makers C26348 and S47656 (Deng et al., 2006), where *Pi9*/*Pi2*, *Pi26(t)* also located, and *Pi25(t)* was mapped on a neighbor region (Liu et al. 2002; Wu et al. 2005).

We further performed fine mapping of *Pigm* with a new mapping population with a total of 1556 susceptible individuals derived from the cross of GM4 and Maratelli which showed rich polymorphism within the mapping region, and narrowed down *Pigm* between the two flanking markers C26348 and S47656 (Fig. 1). Furthermore, three markers, S29742, PC22705, C24901, were identified to co-segregate with *Pigm* (Fig. 1). In addition, our further mapping with other 5 pairs of S and R DNA pools to other 5 different isolates confirmed that the *Pigm* locus confers resistance to different isolates (races). These results indicate that the *Pigm* locus confers broad-spectrum resistance against all isolates *M. oryzae* tested in GM4 (Deng et al. 2006).

A contig map spanning the *Pigm* locus was constructed with BAC/PAC clones of Nipponbare (Fig. 1). As a result, *Pigm* was localized in a 70-kb interval of PAC AP005659 flanked by the markers C5483 and C0428 (Fig. 1), which contains a gene cluster containing 6 predicated NBS-LRR resistance genes according to the sequences of Nipponbare (International Rice Genome Sequencing Project 2005) (Fig. 1), where *Pi9*/*Pi2* also located (Liu et al. 2002; Qu et al. 2006). To clarify the

Fig. 1 (continued) Genetic and physical mapping of the *Pigm* locus. (**A**) Preliminary genetic map of Pigm on chromosome 6 with 306 individuals. (**B**) A contig map spanning the *Pigm* locus based on Nipponbare BAC or PAC clones, with the chromosome orientation indicated. The numbers in parentheses above the map are the numbers of recombinants detected between the corresponding markers and the *Pigm* locus. The numbers below the map indicated positions of the corresponding markers on chromosome 6 based on sequenced map of IRGSP (International Rice Genome Sequencing Project, 2005). The BAC and PAC clones of Nipponbare anchored by the corresponding markers are shown. The *dashed lines* designate the relative positions of the corresponding markers in BAC or PAC clones. (**C**) Putative *R* genes and sequence similarity of the corresponding *Pigm* locus in the genomes of japonica Nipponbare and *indica* 9311. The GenBank accession numbers for R1 to R6 are Os06g17880, Os06g17900, Os06g17920, Os06g17930 Os06g17950 and Os06g17910, respectively. R6 is a pseudogene in Nipponbare and was not found in 9311 because of sequencing gap (indicated by *lines*). (**D**) Physical mapping of *Pigm* based on GM4 BAC clones

gene arrangement in the *Pigm* cluster, we constructed GM4 genome BAC library and sequenced the BAC covering the *Pigm* locus. Eventually the *Pigm* locus was delimited to a 120-kb region between markers p24209 and s48590, co-segregating to markers M18849 and S26205 (Fig. 1). Gene predication and sequence alignment showed that there are 10 NBS-LRR candidate *R* genes in the region panning the *Pigm* locus. It suggested that gene duplication took place during the evolution of the *Pigm* locus. Functional verification of the *Pigm* candidates is under way.

3.3 Pigm Confers Broader Resistance than Alleles Pi2 and Pi9

Two other known broad-spectrum resistance genes, *Pi2* and *Pi9*, were also located in the same region of chromosome 6 (Qu et al. 2006, Zhou et al., 2006), we then wanted to determine the linkage of the three genes. We constructed the F_2 populations of GM4 × C101A51(*Pi2*) and GM4 × 75-1-127(*Pi9*) with 545 and 717 individuals, respectively. Allelism test showed that all F_2 individuals were resistant, no susceptible plant segregated (Table 1). Therefore, we speculated that *Pigm, Pi2* and *Pi9* are allelic. We developed breeding line MP3 from the backcross program (BC4F1) with the recurrent susceptible parent Maratelli, which contains *Pigm* by marker-assistant selection. The inoculation result showed that MP3 and GM4 conferred the same resistance to 18 blast isolates, a spectrum broader than the allelic genes *Pi2, Pi9, Piz* and Piz^t (Table 2).

In order to make use of *Pigm* in rice breeding for blast resistance, a number of PCR-based markers for *Pigm* were developed, which have been proven to be an

Table 2 Comparison of resistance spectrum of *Pigm* and $Pi9/Pi2/Piz/Piz^t$

isolates	GM4	*Pigm*	*Pi9*	*Pi2*	*Piz*	Piz^t	Maratelli
CHL685	R	R	R	S	ND	S	S
CHL541	R	R	R	S	S	MS	S
101/1/1	R	R	R	S	ND	R	S
101/4/8	R	R	R	S	ND	S	S
CH12	R	R	S	S	MR	S	S
CH199	R	R	S	S	MS	R	S
ZJ72	R	R	R	S	ND	R	S
99-30-1	R	R	MR	MR	ND	R	S
CH97	R	R	R	S	ND	R	S
CH227	R	R	S	S	S	R	S
CH131	R	R	MS	R	ND	R	S
CH188	R	R	S	S	MS	R	S
CH102	R	R	R	S	ND	R	S
CH14	R	R	S	S	S	R	S
01–19	R	R	R	R	R	R	S
01–12	R	R	R	MR	MR	R	S
GUY11	R	R	R	S	R	S	S
CH16	R	R	MR	S	R	S	S

R: resistant MR: moderate resistant; S: susceptible; MS: moderate susceptible; ND: No detected.

Table 3 Resistance spectrum of different breeding lines inoculated with 25 blast isolates

isolates	GM4	Z1	Z2	Z3	Z4	Z5	Z6	YFZ
CH99181	0	0	0	0	0	0	0	5
CH0656	0	0	0	0	0	0	0	5
CH0452	0	0	0	0	0	0	0	5
CH01151	0	0	0	0	0	0	0	4
CH05133	0	0	0	0	0	0	0	5
CH02183	0	0	0	0	0	0	0	5
CH07232	1	1	1	1	1	1	1	5
CH07131	0	0	1	1	0	0	0	5
CH99301	0	0	0	0	0	0	0	5
CH0613	0	0	0	0	0	0	0	4
CH0591	0	0	0	0	0	0	0	5
CH05201	0	0	0	0	0	0	0	5
CH035	0	0	1	0	0	0	1	5
CH02182	0	0	0	0	0	0	1	5
CH0332	0	0	0	0	0	0	0	4
CH0721	0	1	0	0	0	0	0	4
CH01060	0	0	0	0	0	0	0	4
CH0514	0	0	2	0	0	0	0	5
CH20002	0	0	0	2	2	2	0	5
CH0551	2	1	2	2	2	2	2	5
CH07143	0	0	0	0	0	0	0	5
CH0214	0	0	0	0	0	0	0	5
CH0211	0	2	0	1	1	0	0	5
CH07162	0	0	2	0	0	0	0	5
CH011621	0	0	0	0	0	0	0	5

Z1: Y6547/GM4//GM4-1 Z2: Y6547/GM4//GM4-2 Z3: Y6547/GM4//GM4-3
Z4: RHT461/GM4-1 Z5: RHT461/GM4-2 Z6:GM4/R461 YFZ: Yuanfengzao.

efficient tool for breeding of *Pigm*-conferred resistance, based on this, some elite resistant lines have been obtained in our hybrid rice breeding program (Table 3).

4 Conclusion

Genetic analysis demonstrates that the Chinese rice variety GM4 harbors the *Pigm* locus with broad-spectrum and durable resistance to rice blast. We applied map-based cloning strategy to delimit *Pigm* to a 120-kb interval containing a 10 NBS-LRR gene cluster on Chromosome 6 of the GM4 genome, the structure of the cluster is different from those of other *R* genes *Pi2/Pi9* mapped on the same region. We confirmed that *Pigm* confers broader-spectrum resistance than *Pi2/Pi9/Piz/Pi26/Piz*^{t}. The identification of all these *R* genes in the same cluster and the further understanding of the recognition of corresponding fungal Avr proteins by these R proteins will provide molecular evidence for elucidating broad-spectrum resistance to blast and the evolution of the *R* gene cluster in the rice genome. Our high-resolution genetic map at the *Pigm* locus and a number of CAPS and InDels markers tightly linked to *Pigm* also facilitate marker-assistant selection for broad-spectrum resistance gene in rice breeding.

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Rac GTPase and the Regulation of NADPH Oxidase in Rice Innate Immunity Response

Hann Ling Wong, Tsutomu Kawasaki and Ko Shimamoto

Abstract Reactive oxygen species (ROS) produced by NADPH oxidase play critical roles in various plant growth, development and defense against pathogens. Plant NADPH oxidases are called regulatory burst oxidase homolog or Rboh. Although Rboh has been isolated from numerous plant species, the molecular mechanism of the regulation of its enzymatic activity remains unclear. All *rboh* genes identified to date possess a conserved N-terminal extension that contains two Ca^{2+}-binding EF-hand motifs. This N-terminal region may provide an entry point for Ca^{2+} to participate in ROS signaling. In phagocyte NADPH oxidase, the small GTPase Rac2 plays the role of a key regulator. In plants, Rac GTPases are also involved in regulation of ROS production and resistance to pathogens in rice. This review discusses recent findings that provide key clues to the understanding of molecular regulation of Rboh by Rac GTPase.

Keywords Rac GTPase · NADPH oxidase · Reactive Oxygen Species

1 Introduction

Reactive oxygen species (ROS) are important for diverse roles in signaling and development in plants, such as plant immunity response, cell death, abiotic stress, stomatal closure and root hair development (Baxter-Burrell et al. 2002; Torres et al. 2002; Foreman et al. 2003; Kwak et al. 2003; Yoshioka et al. 2003). One of the sources of ROS is generated by NADPH oxidase genes, also known as *rboh* (*respiratory burst oxidase homolog*), which encode the homologs of the catalytic subunit $gp91^{phox}$ of the mammalian NADPH oxidase. They have been isolated from many plants species, including rice, *Arabidopsis*, tobacco, tomato, and potato (Torres and

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Dangl 2005). ROS cause severe cellular damages at high concentration, therefore its molecular regulation is paramount for plant growth and survival.

2 NADPH Oxidase-Mediated ROS Production

The phagocyte NADPH oxidase complex is the most well studied NADPH oxidase system. The complex consists of gp91^{phox}, p22^{phox}, p47^{phox}, p67^{phox}, p40^{phox} and the small GTPase Rac2 (Babior 2004). Interestingly, genome sequencing of *Arabidopsis* and rice, revealed the absence of the homolog of these subunits, except for *rboh* and *Rac* (also known as *Rop*), suggesting that plant NADPH oxidase may be regulated differently from animals (Torres and Dangl 2005). Furthermore, unlike the mammalian gp91^{phox}, plant Rboh proteins possess an extended N-terminus, which contains two Ca^{2+}-binding EF-hand motifs. Recently, NADPH oxidases (Nox) containing extended N-terminus that harbors Ca^{2+}-binding EF-hands, have been identified in non-phagocytic cells (Geiszt and Leto 2004; Lambeth 2004; Torres and Dangl 2005). However, the molecular regulation of these novel Nox proteins and the function of their N-terminal EF-hand motifs remain unknown.

Arabidopsis and rice and possess ten and nine *rboh* genes, respectively (Torres et al. 2006; Wong et al. 2007). *Arabidopsis rbohD* and *rbohF* are shown to be involved in ROS production during pathogen infection (Torres et al. 2002; 2006). In *Nicotiana benthamiana*, *rbohA* and *rbohB* are involved in ROS production and contribute to resistance against *Phytophthora infestans* infection (Yoshioka et al. 2003). Despite of increasing evidences pointing to the importance of Rboh-generated ROS in various aspects of signaling and development in plants (Apel and Hirt 2004; Gapper and Dolan 2006), the molecular mechanism of the regulation of plant Rboh eluded discovery for a long time (Apel and Hirt 2004; Torres and Dangl 2005). Recent studies are providing key clues to the elucidation the regulatory mechanism. Parts of this puzzle came from the studies on Rac/Rop, calcium dependent protein kinase (CDPK), and the analysis of the N-terminal region of Rboh.

3 The Role of Rac GTPases in the Regulation of NADPH Oxidase

Small GTPase Rac/Rop is a key regulator of signal transduction in plants. They function as molecular switches cycling between GTP-bound active and GDP-bound inactive forms. *Arabidopsis* and rice possess 11 and 7 Rac/Rop genes, respectively. Rac GTPases are known to interact with multiple effectors, regulating cellular processes, such as actin dynamics, calcium and hormone signalings, ROS production, and gene expression (Yang and Fu 2007). Rac/Rop becomes a prime candidate as a regulator of Rboh by default due to the absence of other homologs of the mammalian Nox subunits.

In rice, overexpression of a constitutively active (CA) form of Rac1 has been shown to enhance pathogen-associated molecular patterns (PAMPs)-induced ROS production, while overexpression of the dominant-negative (DN) form of Rac1 reduces the ROS level (Kawasaki et al. 1999; Ono et al. 2001; Suharsono et al. 2002). Morel et al. (2004) showed that Rac regulation of rbohD is responsible for oxidative burst in elicited tobacco cells. *In vitro* study using *Arabidopsis* cell extracts showed that the GTP-bound Rop2 protein increases ROS production, while the GDP-bound form decreases it, suggesting a direct role of Rop GTPase in ROS production (Park et al. 2004). In addition, a study on Rop GTPase activating protein 4 (*RopGAP4*), an negative regulator of Rop, indicates that Rop is involved in regulation of ROS production in *Arabidopsis* response to oxygen deprivation (Baxter-Burrell et al. 2002). Taken together, these studies provide strong evidence for Rac/Rop as a positive regulator of Rboh-mediated ROS production.

However, in phagocyte, Rac2 activates $gp91^{phox}$ through $p67^{phox}$, which acts as an adaptor in between the two proteins (Babior 2004). But, the homolog of $p67^{phox}$ is not found in plants. Therefore, the regulation of plant NADPH oxidase should be different from the phagocytic NADPH oxidase $gp91^{phox}$. Recently, using yeast two-hybrid assay, we found that rice Rac1 directly interacts with a substantial part of the N-terminal region of Rboh, including the two EF-hand motifs. This finding is supported by further studies using *in vitro* pull-down assay, NMR titration experiment, and *in vivo* fluorescence resonance energy transfer (FRET) microscopy. The FRET analysis also suggests that cytosolic Ca^{2+} concentration may regulate Rac-Rboh interaction in a dynamic manner. Furthermore, transient co-expression of rice Rac1 and rbohB enhances ROS production in *Nicotiana benthamiana*, suggesting that direct Rac-Rboh interaction may activate NADPH oxidase activity in plants (Wong et al. 2007).

4 A Model of Plant NADPH Oxidase Regulation

Previous studies have implicated the involvement of Ca^{2+} in ROS signaling in plants. How Ca^{2+} signaling integrates into Rac/Rop and ROS signaling to modulate plant defense response and development is an intriguing question. Cytosolic Ca^{2+} accumulation has been shown to be a prerequisite for the initiation of oxidative burst (Blume et al. 2000; Grant and Loake 2000; Sagi and Fluhr 2001; Kurusu et al. 2005). However, addition of the calcium ionophore alone is not sufficient to stimulate ROS production (Jabs et al. 1997; Bindschedler et al. 2001). In an unexpected twist, our yeast two-hybrid assay and *in vivo* FRET analysis suggest that Ca^{2+} may inhibit Rac-Rboh interaction. This inhibition of Rac-Rboh interaction is dependent on the EF-hand motif of Rboh and the inhibition is abrogated by a mutation in the EF-hand motif.

A possible explanation on the dual role of Ca^{2+} as a positive and negative regulator is that it acts dynamically by asserting a positive role through CDPK in the initial stage of Rboh-mediated ROS production and subsequently, as ROS

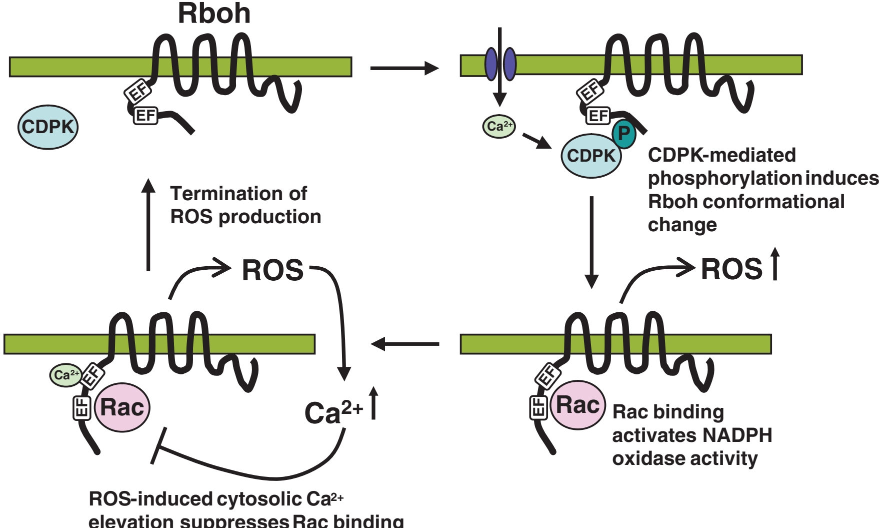

Fig. 1 Model of Rboh regulation by Rac GTPase. The model shows the initial cytosolic Ca^{2+} influx activates CDPK, which phosphorylates the N-terminal region of Rboh, leading to a conformation change that allows Rac GTPase binding and activation of ROS production. Subsequently, the ROS produced may activate cytosolic Ca^{2+} elevation, which inhibits Rac binding, thus terminating the ROS production

are produced, it acts negatively by directly binding to the EF-hand motif of Rboh (Fig. 1). Recently, Kobayashi et al. (2007) found that regulates Rboh-mediated ROS production by phosphorylating the N-terminal region of Rboh. Interestingly, the study also indicates that phosphorylation of Rboh alone is not sufficient for full activation of Rboh. At the initial stage of the oxidative burst, CDPK, in a Ca^{2+}-dependent manner, may sensitize Rboh for activation by phosphorylating the N-terminal region of Rboh, thus inducing a conformational change that release Rboh from an auto-inhibitory state.

The conformational change may expose the N-terminal of Rboh for the interaction with Rac GTPase. Rac binding may stimulate Rboh activation for ROS production. Subsequently, the increase in ROS may induce a second phase of cytosolic Ca^{2+} accumulation by stimulating the opening of plasma membrane calcium channels (Pei et al. 2000). Alternatively, the persistent cytosolic Ca^{2+} accumulation may be caused by a Ca^{2+}-activated Ca^{2+} release mechanism, resulting in the release of Ca^{2+} from internal stores (Ward and Schroeder 1994).

Finally, the cytosolic Ca^{2+} level may attain a threshold that inhibits the Rac-Rboh interaction, thereby terminating ROS production. In effect, the subsequent cytosolic Ca^{2+} elevation may serve as a negative feedback mechanism to dynamically modulate the length of time and possibly the intensity of oxidative burst. This feedback mechanism may operate to restrict cell death at infection sites (Torres and Dangl 2005).

5 Future Prospects

Recent studies have shown that Rac/Rop GTPases are key regulators in cellular signal transduction. The role of Rac in the regulation of Rboh and ROS signaling is becoming clear. However, numerous questions remain to be answered. The spatio-temporal dynamics of Rac activation, Rboh conformation change, calcium, and ROS levels, need to be monitored *in vivo*. Recent advances in bioimaging technologies, such fluorescent probes and FRET biosensors, may help to provide the answers for some of these questions.

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Rice Guanine Nucleotide Exchange Factors for Small GTPase OsRac1 Involved in Innate Immunity of Rice

Tsutomu Kawasaki, Keiko Imai, Hann Ling Wong, Yoji Kawano, Keita Nishide, Jun Okuda and Ko Shimamoto

Abstract Small GTPase OsRac1 is a key regulator for induction of immune responses in rice. Activation of OsRac1 induces NADPH oxidase-mediated reactive oxygen species (ROS) production, PR gene expression, production of antimicrobial compounds, and lignification, which result in enhanced resistance to *Magnaporthe oryzae* and *Xanthomonas oryzae*. Inhibition of OsRac1 function suppresses the hypersensitive response induced by avirulent *M. oryzae*. Thus, it is likely that OsRac1 plays important roles in R protein-mediated resistance and basal resistance. However, how OsRac1 is activated during immune response remains unknown. Recently, a new type of guanine nucleotide exchange factor (GEF) for Rac/Rop GTPase has been found in plants, termed PRONE-type GEF. We found eleven PRONE-type RacGEFs in rice, which may be involved in regulation of OsRac1 in innate immunity response. Recently, the PRONE-type GEF was found to interact with receptor-like protein kinase similar to R-proteins and PAMPs receptors such as Xa-21, Pi-d2, FLS2 and EFR, suggesting that the OsRacGEFs may regulate both PAMPs- and R protein-mediated disease resistance through activation of OsRac1 in rice.

Keywords Rac · Small GTPase · Guanine nucleotide exchange factor

1 Introduction

Plants develop immune systems to inhibit infection by pathogens. These immune systems are initiated by both general recognition of pathogen-associated molecular patterns (PAMPs) and the specific recognition of particular–encoded molecules through the action of plant disease resistance (R) protein (Jones and Dangl 2006). The recognition of pathogens triggers a series of defense responses, including ROS

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production, pathogenesis-related (PR) gene expression, and production of antimicrobial compounds. These responses result in effective restriction of pathogen growth at the infection site. However, the molecular mechanisms how a series of defense responses are induced after the pathogen recognition was not well understood.

The small GTPase OsRac1 functions as a key switch to induce defense response in rice. However, how OsRac is regulated during defense response remains unknown. In this study, we focus on the role of OsRac1 in the defense responses, especially lignin biosynthesis. We also introduce the guanine nucleotide exchange factors (GEFs) for Rac/Rop GTPase, and elaborate involvement of Rac/Rop GEFs in innate immune responses.

2 The Roles of Small GTPase OsRac1in Innate Immunity Responses

Plant Rac/Rop small GTPases constitute a unique subfamily of the Rho family of small GTPase. Rice contains seven members of Rac family (OsRac1-OsRac7), whereas 11 members (Rop1-Rop11) are found in Arabidopsis. The function of OsRac1 in immune responses has been extensively studied. Activation of OsRac1 induces ROS production in rice, which is inhibited by an inhibitor for flavin-containing phagocytic NADPH oxidase (Kawasaki et al. 1999), indicating that OsRac1 regulates the NADPH oxidase-mediated ROS production. Recently, we found that OsRac1 directly interacts with plasma membrane-localized NADPH oxidase and regulates ROS production (Wong et al. 2007). The ROS production was accompanied with a series of immune responses including PR gene expression, production of antimicrobial compounds, cell death, and lignification (Ono et al. 2001; Wong et al. 2004).

Transgenic rice plants expressing constitutively active (CA) mutant of OsRac1 induce a series of immune responses, which result in enhanced resistance to virulent race of *M. oryzae* and *X. oryzae* (Ono et al. 2001). On contrary, the hypersensitive response induced by avirulent *M. oryzae* is suppressed by expression of dominant-negative (DN) form of OsRac1 (Ono et al. 2001) or silencing of OsRac1 by RNAi (Chen et al. unpublished results). Thus, it is likely that OsRac1 plays important role in R protein-mediated resistance and basal resistance. Furthermore, OsRac1 was found to interact with important immune components including RAR1 and HSP90 (Thao et al. 2008). RAR1 and HSP90 are known to control stability of NBS-LRR type R-proteins, suggesting that OsRac1 may be involved in defense signaling mediated by R-proteins and PAMPs receptors.

3 OsRac1 Regulates Lignin Biosynthesis

Deposition of lignin on cell wall is one of the innate immune responses. Lignin, which is polymerized through peroxidase activity using H_2O_2 in the cell wall, presents an undegradable mechanical barrier to most pathogens. Rice cinnamoyl-CoA reductase 1 (OsCCR1), a key enzyme for synthesis of monolignols in lignin

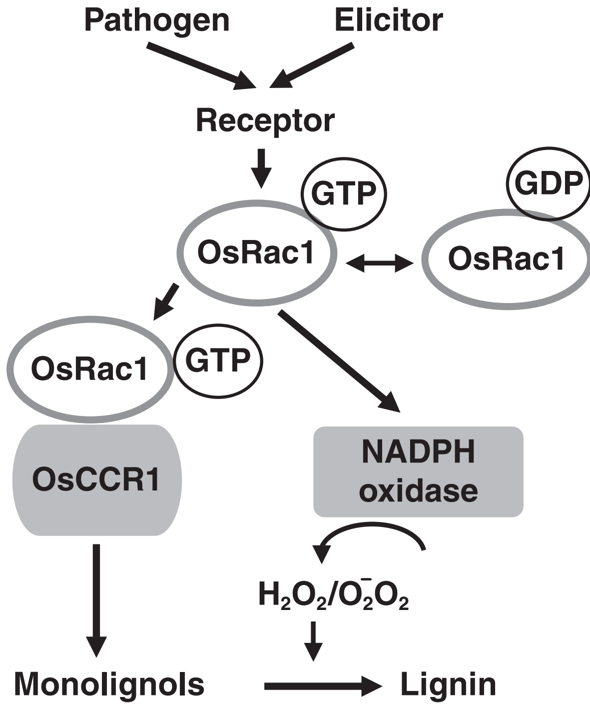


Fig. 1 Proposed model for the dual function of OsRac1 in lignin biosynthesis. OsRac1 activates CCR activities, which results in enhanced production of monolignols. OsRac1 also activates NADPH oxidase during the defense response, which leads to the increased production of H_2O_2. As a consequence, monolignols are efficiently polymerized by peroxidase with H_2O_2

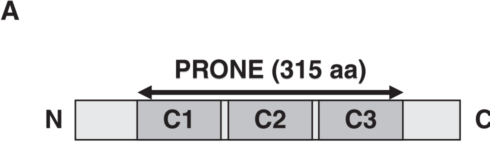
biosynthesis, has been identified as an effector of OsRac1 (Kawasaki et al. 2006). OsCCR1 is specifically expressed during defense response, indicating that OsCCR1 participates in lignin biosynthesis in innate immune response. OsCCR1 specifically interacts with CA-OsRac1, but not DN-OsRac1, indicating that the interaction between OsCCR1 and OsRac1 is GTP-dependent. Interestingly, the interaction of OsCCR1 with OsRac1 drastically activates OsCCR1 activity *in vitro*, which is supported by the fact that transgenic cell cultures expressing the CA-OsRac1 accumulate lignin through enhanced CCR activity (Kawasaki et al. 2006). As mentioned above, OsRac1 also stimulates NADPH oxidase-dependent ROS production that is required for polymerization of monolignol on the cell wall (Wong et al. 2007). This finding suggests that OsRac1 controls lignin synthesis through regulation of both NADPH oxidase and OsCCR1 activities during innate immune response in rice (Fig. 1).

4 Plant Guanine Exchange Factors for Rac/Rop GTPase

As mentioned above, OsRac1 GTPase plays very important roles in innate immune responses. However, how OsRac1 is activated during immune response remains to be identified. Plant Rac/Rop GTPases belong to Rho family of GTPase. So far, many GEFs for Rho family have been isolated in animal. Most of Rho GEFs contain Dbl

homology (DH) domain and pleckstrin homology (PH) domain, which referred to as DH-PH type GEF (Schmidt and Hall 2002). There are 60, 18, 6, and 23 DH-PH type GEF genes in human, *C. elegans*, yeast, and Drosophila, respectively. Interestingly, in silico searches indicate that plants do not contain any DH-PH type GEFs. Therefore, different types of GEFs have been considered to regulate the activity of Rac/Rop GTPases in plants. Recently, a new type of Rac/Rop GEF has been identified in Arabidopsis. The GEF contains highly conserved region composed of 315 amino acids, referred to as PRONE (*P*lant specific *Ro*p *n*ucleotide *e*xchanger) (Fig. 2A) (Berken et al. 2005; Gu et al. 2006). The PRONE domain is plant specific, which was not found in other organisms.

The PRONE-type Rac/Rop GEFs have the variable regions at N- and C-terminus in addition to central PRONE domain (Fig. 2). The PRONE domains were shown to exhibit the GEF activity toward Rop GTPase in Arabidopsis (Berken et al. 2005; Gu et al. 2006). The N- and C- terminal variable regions function as inhibitory domain for activation of RopGEFs (Gu et al. 2006; Zhang and McCormick 2007). The intramolecular interaction of the inhibitory domains with other regions inhibits the own GEF activity (Gu et al. 2006). Intramolecular interaction of the DH-PF type Rho GEFs is known to be released by phosphorylation of the inhibitory domain, or interaction of other factors to the inhibitory domain (Schmidt and Hall 2002). Previously, it was reported that the PRONE type RopGEF was also phosphorylated (Kaothien et al. 2005), suggesting that the RopGEF activity may be regulated in similar manner as DH-PH type GEF.



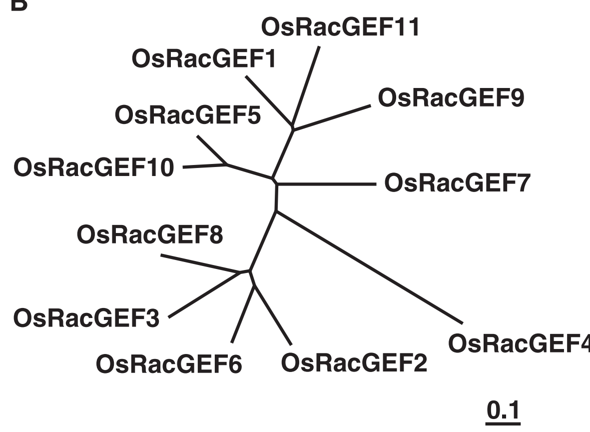


Fig. 2 Rice PRONE-type RacGEFs. (**A**) Structure of PRONE-type Rac/Rop GEF. The GEF contains highly conserved domain, referred to as PRONE. The PRONE domain is composed of three subdomains (C1, C2, and C3). The N- and C-terminal regions outside the PRONE domain are highly variable. (**B**) Phylogenetic tree of OsRacGEFs. Phylogenetic tree was obtained using Clustal W

Rice contains 11 PRONE-type RacGEFs (Fig. 2), whereas there are 14 PRONE GEFs in Arabidopsis. The PRONE domains of rice RacGEFs (OsRacGEFs) are highly conserved, although the N- and C-terminal regions are diverse in sequence and length. The PRONE domains are largely classified into two groups based upon differences of the amino acid sequences as reported by Zhang and McCormick (2007). All OsRacGEFs interacted with OsRac1 *in vitro*, although the affinities are different among OsRacGEFs (Imai et al. unpublished result). In addition, most of OsRacGEFs exhibited significant GEF activity toward OsRac1 (Imai et al. unpublished result), suggesting that most of OsRacGEFs have abilities to activate OsRac1. To identify the OsRacGEFs that function during defense responses, we analyzed the expression of OsRacGEFs after treatment of sphingolipid elicitors (SE) that was prepared from plasma membrane of rice blast fungus. Activation of defense responses by SE occurs through the function of OsRac1, because most of SE-inducible proteins are induced by CA-OsRac1 (Fujiwara et al. 2006) and DN-OsRac1 inhibits SE-mediated defense response (Suharsono et al. 2002). We found that expression of some of OsRacGEFs was affected by treatment of SE (Imai et al. unpublished result), suggesting that some OsRacGEFs may be regulated at transcription level during defense response.

The PRONE-type Rac/RopGEFs were initially identified form tomato by yeast two-hybrid screening by using the kinase domain of receptor-like kinase (RLK) as a bait (Kaothien et al. 2005). Furthermore, tomato RopGEF is phosphorylated *in vivo*, suggesting that the PRONE-type RopGEFs may be phosphorylated and activated by RLK. Recently, it was reported that Arabidopsis RopGEF12, which is expressed specifically in pollen, interacts *in vivo* with pollen receptor like kinase (AtPRK2a) at the C-terminal domain (Zhang and McCormick 2007). The C-terminal domain of RopGEF12 has the inhibitory activity for the own GEF function and contains putative phosphorylation sites. A phospho-mimicking mutation within the C-terminal domain indicates the enhanced activity of RopGEF12, suggesting that activation of RopGEF12 is regulated by phosphorylation of its C-terminal inhibitory domain (Zhang and McCormick 2007). Thus, it is likely that PRONE-type GEF may function as a signal transmitter from RLK to the downstream through the function of Rac/Rop GTPase.

Some of RLKs are known to function as R-proteins and PAMPs receptors such as Xa21, Pi-d2, FLS2, ERF, and CERK1 (Dardick and Ronald 2006; Miya et al. 2007). Recently, the MAP kinase pathway was found to be activated downstream of these RLKs during defense responses (He et al. 2006; Miya et al. 2007). However, it is also possible that Rac/Rop GTPase transmits the signals from RLK to the downstream factors through Rac/RopGEFs in innate immunity responses. In fact, accumulating evidences indicate that Rac GTPase plays important roles in PAMPs and R-protein mediated signaling. Whether Rac/Rop or Rac/RopGEF interacts with RLK in innate immunity responses is an interesting issue.

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Role of Ethylene, Abscisic Acid and MAP Kinase Pathways in Rice Blast Resistance

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Abstract A combination of genetic, molecular, biochemical and pathological approaches have been taken to elucidate the defense signaling pathways leading to rice blast resistance and susceptibility. Using transgenic rice lines defective in salicylic acid (SA) or jasmonic acid (JA) pathways, we have previously shown that SA is not an effective signal molecule in rice but acts as a constitutive antioxidant to protect rice plants from the pathogen-induced oxidative damage. On the other hand, JA pathway is involved in mediating rice defense gene expression and blast resistance. Recently, increasing evidence suggests that ethylene (ET) biosynthesis is important for rice blast resistance. Based on the analysis of transgenic rice lines defective in ET, abscisic acid (ABA), or mitogen-activated protein (MAP) kinase pathways, we have demonstrated that ET and its antagonistic interaction with ABA plays a crucial role in rice blast resistance. Furthermore, a stress-responsive MAP kinase was found to mediate the cross-talk between ABA and ET pathways and inversely regulate rice blast resistance and abiotic stress tolerance.

Keywords Defense pathways · Host resistance · Protein kinase · Signal transduction

1 Introduction

Rice blast disease, caused by *Magnaporthe oryzae*, is regarded as the most feared disease of rice worldwide because of its seemingly sporadic and unpredictable outbreaks that frequently result in drastic yield losses. The breeding strategy for rice blast resistance has been mainly based on the introduction and pyramiding of race-specific, major resistance (*R*) genes. While vertical resistance mediated by *R*

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genes is highly effective, it is often unstable due to race-change mutations in the rice blast fungus. An alternative approach to combat rice blast disease is the utilization of horizontal or partial resistance which is usually unaffected by the variation of pathogen races and capable of reducing disease development in spite of a compatible infection. In rice, horizontal or field resistance to blast infection has long been observed in rice plants grown under flood or anaerobic conditions (Kahn and Libby 1958; Kim et al. 1985; Hemmi et al. 1941; Lai et al. 1999). On the other hand, rice plants growing under low soil moisture (such as drought stress and upland culture conditions) are more susceptible to rice blast disease (Bonman et al. 1988; Gill and Bonman 1988). It is known that rice plants growing under low soil moisture produce increased levels of ABA but low levels of ET (Van der Straeten et al. 2001). By contrast, flooded rice plants (under hypoxia condition) have low levels of ABA but accumulate high levels of ET by activation of its biosynthetic genes and physical entrapment of the gas due to its reduced diffusion rate in water (Kende et al. 1998). Recently, increasing evidence from our lab and other groups suggests that ET and its antagonistic interaction with ABA may play a prominent role in vertical and horizontal resistance to blast infection (Zhou et al. 2006; Iwai et al. 2006; Singh et al. 2004). Furthermore, the MAP kinase cascade may mediate the cross-talk between the ET and ABA pathways and regulate rice blast resistance.

2 Ethylene Pathway is Important for Rice Defense Gene Expression and Blast Resistance

As the simplest unsaturated hydrocarbon, ET (C_2H_4) is involved in diverse metabolic and developmental processes such as seed germination, fruit ripening and senescence (Bleecker and Kende 2000). Depending on the plant-pathogen combination and specific environmental conditions, ET may act as a positive or negative modulator of disease resistance (Broekaert et al. 2006; van Loon et al. 2006). In *Arabidopsis*, the ET-insensitive mutant *ein2* develops only minimal disease symptoms as the result of enhanced disease resistance to virulent *Pseudomonas syringae* pv. *tomato* or *Xanthomonas campestris* pv. *campestris* (Bent et al. 1992). In contrast, the *ein2* mutant was more susceptible to the necrotrophic fungus *Botrytis cinerea*, but not to avirulent strains of either *Peronospora parasitica* or *Alternaria brassiccola* (Thomma et al. 1999). Several other studies using *Arabidopsis*, tobacco and tomato also demonstrated that ET, which often acts in concert with JA, generally contributes to host resistance against necrotrophic, but not biotrophic pathogens (van Loon et al. 2006). However, relatively little is known about the role of ethylene biosynthesis or signal transduction in rice disease resistance.

It has been proposed that flood or hypoxia-induced ET biosynthesis in rice is critical for conferring field or horizontal resistance to the blast disease in the Southern U.S (Singh et al. 2004). Application of AVG (amino-ethoxyvinylglycine hydrochloride, an ET biosynthesis inhibitor) increased blast disease severity and negated the flood- induced resistance in rice plants. Conversely, application of ethephon (2-chloroethylphosphonic acid, an ET generator) significantly increased rice blast

resistance in disease susceptible cultivars (Singh et al. 2004). Previously, exogenous ET treatment was shown to induce defense gene expression and the accumulation of phenylpropanoid-derived phytoalexin sakuranetin (Agrawal et al. 2000; Jwa et al. 2006; Nakazato et al. 2000). Recently, Iwai et al. (2006) found that ET biosynthesis in rice seedlings was markedly induced during the resistance interaction at 48 hours post-infection with *M. oryzae*. Interestingly, the elevated ET levels coincided with the appearance of the hypersensitive response (HR) and defense gene expression. Treatment of rice seedlings with aminooxyacetic acid, an ACC synthase inhibitor, suppressed the ET emission and increased blast lesion size and disease susceptibility. However, treatment with 1-methylcyclopropene and silver thiosulfate (inhibitors of ET signal transduction) did not affect HR formation in the resistance response. The authors suggest that cyanide, the coproduct of ET biosynthesis, may play an important role (Iwai et al. 2006).

To determine the role of ET signaling in rice blast resistance, it is essential to generate and characterize rice transgenic lines defective of ET signal transduction. In *Arabidopsis*, EIN2 (ETHELENE INSENSITIVE 2) is a central component of ET signal transduction, whose mutation leads to complete insensitivity to ethylene (Alonso et al. 1999). In contrast to *Arabidopsis* that contains a single copy of *EIN2*, rice carries two *EIN2*-like genes. Using the RNAi approach, we have generated transgenic rice lines with strong suppression of single or both *OsEIN2* genes. These single (*ein2a* or *ein2b*) and double (*ein2*a+b) mutant lines exhibited insensitivity to ET, delayed flowering and relatively poor seed setting. In addition, ACC-induced expression of *PR1b, PR5* and *PR10* genes was significantly reduced in the *OsEIN2b* RNAi lines, suggesting that ET signaling is important for defense gene activation. However, ET biosynthesis was not significantly affected in *OsEIN2b* RNAi lines.

Similar to *Arabidopsis ein2* mutant which is supersensitive to ABA treatment, rice *OsEIN2b* RNAi lines were hypersensitive to ABA as demonstrated by strong inhibition of seed germination in the presence of ABA. Furthermore, ABA-responsive genes (e.g., *LIP9* and *salT*) were induced much higher in the *OsEIN2b* RNAi lines. Disease assays using a moderately virulent blast isolate demonstrated that the *OsEIN2b* RNAi lines were more susceptible to blast infection as shown by increased fungal growth and more severe disease symptoms. RNA blot analysis showed that *PR1b*, *PR5* and *PR10* expression was reduced in *OsEIN2b* RNAi lines at 2–6 days post-infection, which may result in weakened defense response against the fungal infection. Interestingly, the same *OsEIN2b* lines were much more tolerant to drought, salt and cold stress, indicating that *OsEIN2b* mediates the cross-talk between ET and ABA signaling and inversely regulates disease resistance and abiotic stress tolerance in rice.

3 Abscisic Acid Interacts Antagonistically with Ethylene and Negatively Modulates Rice Blast Resistance

ABA is well characterized as a secondary signal molecule during plant growth and development, wounding and abiotic stress responses to salinity, drought and low temperature. However, relatively little is known about the role of ABA in

plant-pathogen interactions (Mauch-Mani and Mauch 2005). In rice, a couple of studies link ABA as a negative regulator of blast resistance. For example, exogenous application of ABA and low temperature increases the susceptibility of blast susceptible cultivars (Koga et al. 2004; Matsumoto et al. 1980). In addition, inhibition of ABA synthesis by the application of fluorine (an inhibitor of ABA biosynthesis) prevents cold-induced blast susceptibility (Koga et al. 2004).

Up to date, the molecular and biochemical mechanisms associated with ABA-mediated blast susceptibility is poorly characterized. Koga et al. (2004) suggests that the ABA pathway functions independently of vertical resistance because the exogenous application of ABA to blast resistant rice cultivars does not compromise major gene-mediated disease resistance. We have found that exogenous application of ABA drastically reduced endogenous ET levels in rice and significantly increased blast susceptibility in a compatible interaction. In addition, suppression of *OsEIN2b* in rice resulted in ABA hypersensitivity, reduced defense gene expression and increased disease susceptibility. By antagonistically interacting with ethylene, therefore, ABA appears to modulate downstream signaling and defense gene expression associated with horizontal or field resistance to the blast fungus.

4 The MAP Kinase Cascade Mediates Ethylene and Abscisic Acid Cross-Talk and Regulates Rice Blast Resistance

The MAP kinase cascade is involved in a plethora of signaling pathways associated with plant growth and development as well as abiotic and biotic stress responses. Among 17 rice MAP kinase genes, at least half of them are associated with biotic and/or abiotic stress response (Reyna and Yang 2006; Rohila and Yang 2007). OsMPK5, the rice orthologue of *Arabidopsis* AtMPK3, is induced by ABA, pathogen infection and abiotic treatments (Xiong and Yang 2003). Our previous study showed that *OsMPK5* overexpression (OX) lines exhibited increased tolerance to drought, salt and cold stresses. By contrast, suppression of *OsMPK5* by RNAi reduced abiotic stress tolerance, but led to constitutive expression of *PR* genes and enhanced disease resistance to the fungal and bacterial pathogens. We recently found that *OsMPK5* RNAi lines had a higher level of ET and a lower level of ABA. In contrast to AtMPK3/6 which positively regulates ET biosynthesis in *Arabidopsis*, therefore, OsMPK5 appears to negatively modulate the ET level, but positively regulate the ABA level in rice. The higher levels of ET in *OsMPK5* RNAi lines could be drastically reduced by exogenous ABA treatment, which led to increased disease susceptibility to rice blast fungus. In addition to its effect on ET and ABA levels, OsMPK5 may also regulate ET and ABA signal transduction. Suppression of *OsMPK5* in rice resulted in reduced ABA sensitivity. Furthermore, OsMPK5 was found to directly interact with and phosphorylate OsEIL1, an orthologue of *Arabidopsis* EIN3. These results suggest that OsMPK5 may mediate ET and ABA cross-talk in rice through both hormone levels and their signal transduction.

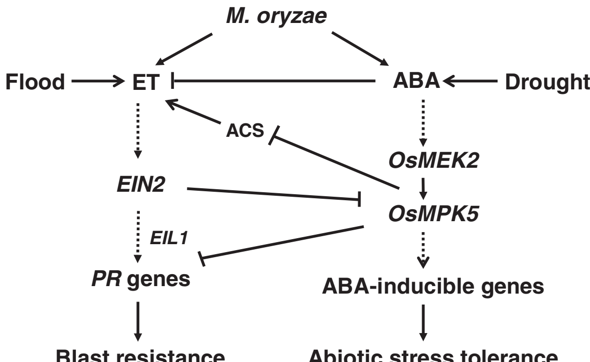

Fig. 1 Antagonistic interaction of ethylene and ABA pathways modulates rice blast resistance and abiotic stress tolerance

5 Concluding Remarks

Emerging evidence suggests that ET and its antagonistic interaction with ABA may play a crucial role in rice blast resistance and abiotic stress tolerance (Fig. 1). During the rice-*M. oryzae* interaction, it is hypothesized that NBS-LRR proteins encoded by major *R* genes may detect the interaction between the fungal effectors and host cell targets, relay the early signal through the MAP kinase cascade and subsequently activate downstream ET-, ABA- and JA-mediated defense pathways. Abiotic stresses such as flood and drought can also activate the MAP kinase, ET and/or ABA signal pathways and influence rice defense responses such as field resistance to blast infection. A better understanding of the interplay among the MAP kinase, ET and ABA pathways may help develop better breeding strategies and cultural practices to control rice blast disease.

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Bacterial Determinants and Host Defense Responses Underpinning Rhizobacteria-Mediated Systemic Resistance in Rice

David De Vleesschauwer and Monica Höfte

Abstract Selected strains of plant growth-promoting rhizobacteria are capable of reducing disease incidence in aboveground plant parts through induction of a defense state commonly referred to as Induced Systemic Resistance (ISR). Compared to the relative wealth of information available in dicotyledoneous plant species, little is known about the molecular and cellular basis of ISR in economically important cereal crops. To gain more insight into the resistance mechanisms underpinning ISR in rice, we analyzed the bacterial determinants and host defense responses associated with induction of blast resistance by the fluorescent pseudomonads *P. aeruginosa* 7NSK2 and *P. fluorescens* WCS374r. Despite triggering similar levels of protection against blast, both rhizobacteria were found to employ remarkably distinct resistance strategies. Whereas 7NSK2 bacteria elicited ISR through secretion of the redox-active pigment pyocyanin, thereby sensitizing the tissue for hyperactivation of HR-like cell death upon challenge attack, establishment of ISR by WCS374r relied on production of the siderophore pseudobactin, a process shown to activate a salicylic acid-independent signaling pathway culminating in the boosted expression of an attacker-induced multifaceted cellular defense response. This apparent flexibility in the molecular processes leading to ISR strongly suggests that rice may be endowed with multiple blast-effective resistance pathways. Further insight into the induced resistance machinery of rice will not only advance our fundamental understanding of the signaling circuitry and targeted defense responses regulating ISR in rice and other cereal crops, but also be instrumental in developing novel strategies for biologically based, economically viable and environmentally sound crop protection.

Keywords Induced systemic resistance · Host defense · *Rhizobacteria*

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1 Introduction

Plants as sessile organisms have evolved a plethora of sophisticated defense strategies to resist potential invasion by microbial pathogens or attack by herbivorous insects. Pre-existing biochemical and physical barriers constitute the first line of defense. Besides constitutive defenses, plants have developed highly sensitive sensory circuits that allow them to recognize invaders as well as coordinated signal transduction pathways that link pathogen perception with targeted defense responses. These pathogen-inducible defenses are part of the plant's basal resistance response and significantly slow down pathogen ingress. However, the effectiveness of this basal resistance can be greatly improved when plants are appropriately stimulated by specific biotic or abiotic elicitors prior to pathogen infection, a phenomenon generally known as induced resistance (Bostock 2005).

A classic example of an inducible plant defense response that has been demonstrated for many plant-pathogen interactions is systemic acquired resistance (SAR). SAR is triggered by localized infection with necrotizing microbes and is manifested throughout the plant upon secondary challenge by otherwise virulent microbes (for review see Durrant and Dong 2004). Colonization of roots with benign plant growth-promoting rhizobacteria (PGPR) leads to a phenotypically similar form of induced resistance, commonly referred to as induced systemic resistance or ISR (van Loon et al. 1998).

Although both rhizobacteria-mediated ISR and pathogen-induced SAR convey protection against a broad spectrum of pathogens, their signal transduction pathways are clearly distinct. Whereas SAR is associated with a local and systemic increase in endogenously synthesized salicylic acid (SA) and a coordinate expression of a battery of defense-related genes, including those encoding pathogenesis-related (PR) proteins (Maleck et al. 2000), rhizobacteria-triggered ISR predominantly functions independently of SA and PR proteins (Pieterse et al. 1996; van Wees et al. 1999). Conversely, analysis of several *Arabidopsis* signaling mutants revealed that ISR triggered by *Pseudomonas fluorescens* strain WCS417 requires intact sensitivity to the plant hormones jasmonic acid (JA) and ethylene (ET) (Pieterse et al. 2000).

In general, ISR is characterized by a faster and stronger activation of basal stress-inducible defense mechanisms in response to pathogen attack rather then a direct activation of defenses (Beckers and Conrath 2007). By analogy with a phenotypically similar phenomenon in animals and humans, this enhanced capacity to express basal defense mechanisms is called "potentiation" or "priming" (Conrath et al. 2002).

Compared to the vast body of information on induced resistance in dicot plants, our understanding of biologically induced defense responses in monocot crops is still rudimentary. Although the synthetic SA analogue benzothiadiazole has been shown to induce SAR in wheat (Gorlach et al. 1996) and disease resistance in rice (Rohilla et al. 2002; Shimono et al. 2007) and maize (Morris et al. 1998), reports about the induction of systemic resistance in monocots using beneficial microorganisms are scarce. These include one in barley, where pre-inoculation with the root-colonizing fungus *Piriformospora indica* was shown to mount resistance to

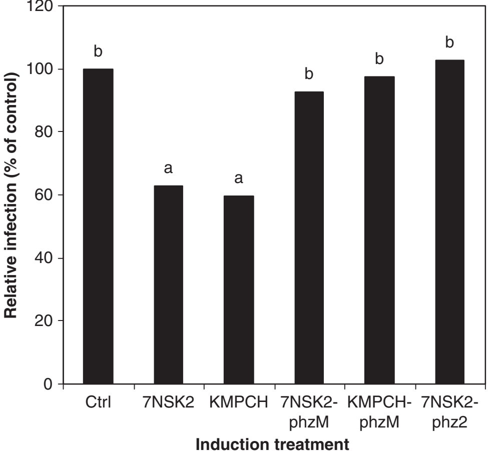
several fungal diseases by stimulating the glutathione-ascorbate cycle (Waller et al. 2005). In rice, colonization of the rhizosphere with the PGPR strains *Pseudomonas fluorescens* PF1 and FP7 enhanced resistance against *Rhizoctonia solani* (Nandakumar et al. 2001). Similarly, Someya et al. (2002, 2005) reported induced resistance to rice blast and sheath blight by the antagonistic bacterium *Serratia marcescens* B2. Nevertheless, no information is yet available regarding the bacterial determinants and molecular mechanisms underpinning PGPR-mediated ISR in rice or other monocots. Aiming to dissect the induced systemic resistance response in rice, we analyzed the bacterial determinants and plant defense responses underlying *Pseudomonas aeruginosa* 7NSK2- and *Pseudomonas fluorescens* WCS374r-triggered ISR in rice.

2 Redox-Active Pyocyanin: Two-Faced Player in *P. aeruginosa* 7NSK2-mediated ISR (De Vleesschauwer et al. 2006)

2.1 Pyocyanin is a Crucial Determinant of 7NSK2-mediated ISR to M. oryzae

In a standardized soil-based assay, root colonization with the PGPR strain *P. aeruginosa* 7NSK2 effectively protected rice leaves against challenge infection with *Magnaporthe oryzae*, the causal agent of the devastating rice blast disease. Because inducing bacterium and challenging pathogens remained spatially separated throughout the experiment, antagonism by direct interactions could be ruled out, demonstrating that 7NSK2-triggered protection is host plant mediated. ISR against *M. oryzae* was manifested as a reduction in the number of susceptible-type blast lesions, thereby resembling the resistance phenotype of quantitative trait loci-governed partial resistance.

In dicot plants, *P. aeruginosa* 7NSK2 elicits ISR through a synergistic interaction of the siderophore pyochelin and the phenazine antibiotic pyocyanin, presumably leading to generation of extremely toxic hydroxyl radicals (Audenaert et al. 2002). In order to identify the bacterial factors operative in triggering systemic resistance to *M. oryzae*, the potency of *P. aeruginosa* 7NSK2 to induce ISR was compared with that of a collection of mutants deficient in the production of pyocyanin and/or pyochelin. Figure 1A shows that the pyochelin-negative mutant KMPCH (also pyoverdin deficient) induced resistance to an extent similar to that induced by the wild-type, thereby excluding an essential role of the siderophores pyoverdin and pyochelin in ISR in rice to *M. oryzae*. Conversely, treatment with the pyocyanin-negative mutants 7NSK2-phzM and KMPCH-phzM no longer caused disease reduction, while i*n trans* complementation of 7NSK2-phzM for pyocyanin production (strain 7NSK2-phzMc) restored the capacity to induce resistance to *M. oryzae*. Hence, these results strongly suggest that the phenazine antibiotic pyocyanin is an essential determinant of 7NSK2-mediated ISR to *M. oryzae*.



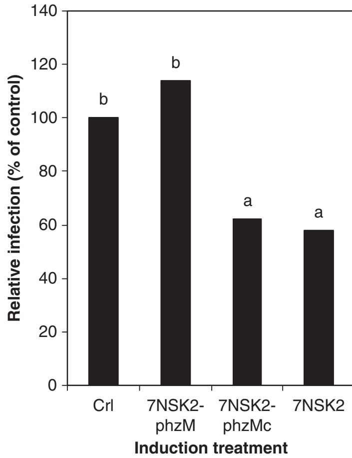


Fig. 1 Influence of root treatment with *Pseudomonas aeruginosa* 7NSK2 and various mutants on rice blast (*Magnaporthe oryzae*) severity. Mutants derived from strain 7NSK2 have the following characteristics: KMPCH (pyoverdin and pyochelin deficient), 7NSK2-phzM (phzM^{-}, nonproducing pyocyanin), KMPCH-phzM (pyoverdin and pyochelin deficient; phzM^{-}, nonproducing pyocyanin) and 7NSK2-phzMc = strain 7NSK2-phzM complemented with functional phzM gene of 7NSK2, restoring pyocyanin production. Bars labeled with the same letter are not significantly different according to non-parametric Kruskal-Wallis and Mann-Whitney comparison tests ($P = 0.05$). (Copyright © by the American Phytopathological Society; reprinted from De Vleesschauwer et al. 2006)

2.2 Pyocyanin Acts as a Negative Regulator of ISR to Rhizoctonia solani

In spite of its resistance-inducing potential against *M. oryzae*, *P. aeruginosa* 7NSK2 proved unable to consistently mount ISR to the sheath blight fungus *R. solani* in several preliminary experiments. These data notwithstanding, we tested the same set of mutant strains as described before in a series of infection assays with *R. solani* as challenging pathogen. Neither the wild-type strain 7NSK2 nor the pyochelin-negative mutant KMPCH significantly reduced sheath blight severity (Fig. 2). Nevertheless, inoculation of the rhizosphere with the corresponding pyocyanin-deficient strains (7NSK2-phzM and KMPCH-phzM) resulted in significantly higher protection levels to *R. solani* compared to wild type-treated and control plants.

2.3 Molecular Insights into Pyocyanin-Induced Blast Resistance

The observation that pyocyanin-deficient mutants, unlike wild-type strains, triggered resistance to *R. solani*, whereas the same mutants lost their ability to mount ISR to *M. oryzae* (Figs. 1A and 2), suggested that the secretion of pyocyanin might account for the differential effectiveness of 7NSK2-mediated ISR to the

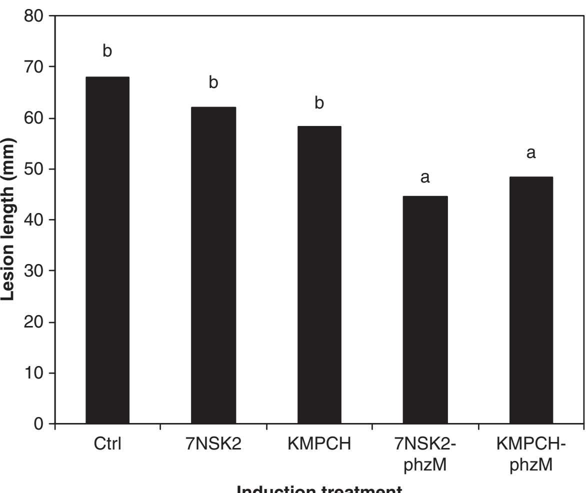


Fig. 2 Pyocyanin acts as a negative regulator of ISR against *R. solani*. For characteristics of parental and mutants strains, see Fig. 1. (Copyright © by the American Phytopathological Society; reprinted from De Vleesschauwer et al. 2006)

latter pathogens. To further explore the role of bacterially produced pyocyanin in 7NSK2-mediated ISR in rice, we isolated pyocyanin from bacterial cultures and applied the purified compound to the roots of hydroponically grown rice seedlings. No signs of phytotoxicity were observed in leaves of plants upon pyocyanin feeding at any of the concentrations tested. In the 25 pM to 100 nM pyocyanin range, ISR to *M. oryzae* was evident for all concentrations tested. However, no significant protection could be observed at 50 μM pyocyanin. Conversely, pyocyanin feeding favored subsequent infection by *R. solani*, irrespective of the applied concentration **(De Vleesschauwer et al. 2006)**. Hence, these data corroborate the results from the bacterial mutant analysis and suggest an ambivalent role of pyocyanin in 7NSK2-mediated ISR.

Because pyocyanin is able to redox cycle under aerobic conditions with resulting generation of superoxide and hydrogen peroxide *in vitro* (Hassan and Fridovich 1980), we asked whether pyocyanin also would be capable of producing ROS in our gnotobiotic system. To this end, we monitored the levels of H_2O_2, which is the major and most long-living reactive oxygen species, both on the roots and in the leaves of hydroponically grown rice seedlings in response to pyocyanin feeding. Roots of rice seedlings treated with 100 nM pyocyanin showed strong DAB staining compared to Hoagland-treated control roots (Fig. 3A,B). However, DAB staining was not observed in the presence of the H_2O_2 scavenger, ascorbic acid, confirming the specificity of the staining (data not shown). The *in planta* accumulation of H_2O_2 was then determined following the titanium (IV) chloride method as described by (Wu et al. 1995). Inclusion of 100 nM pyocyanin in the nutrient solution revealed a transient rise in H_2O_2 levels in systemic leaves at 8 h post-application compared to control plants, followed by decay to control levels (Fig. 3C). A second more pronounced rise in H_2O_2 was observed at 48 h post-treatment and persisted for at least 24 h. Taken together; these data clearly demonstrate the ability of bacterial

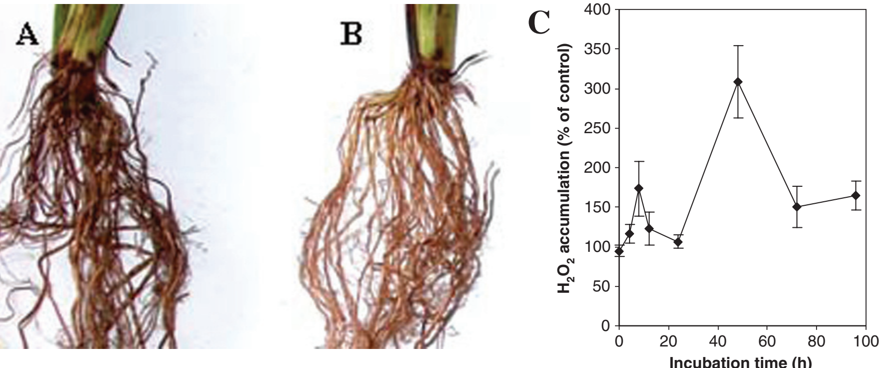


Fig. 3 Induction of H_2O_2-accumulation on local roots and reiterative H_2O_2-microbursts in systemic leaves in response to pyocyanin feeding. Roots of hydroponically grown rice seedlings were immersed for 2 h in nutrient solution (**A**) with or (**B**) without pyocyanin (1 nM), rinsed several times with distilled water and subsequently incubated in DAB solution (1 mg/ml) for 12 h at room temperature. (**C**) H_2O_2 levels in systemic leaves in response to root treatment with pyocyanin. (Copyright © by the American Phytopathological Society; reprinted from De Vleesschauwer et al. 2006)

pyocyanin to interfere with the plant's oxidative machinery leading to local H_2O_2-generation on the root surface coupled to reiterative H_2O_2-microbursts in systemic leaves. This observation prompted us to test whether ROS generated by pyocyanin *in planta* account for the dual role of the latter compound in 7NSK2-mediated ISR. To this end, we investigated the effect of adding H_2O_2-quenching ascorbate to the pyocyanin solution on subsequent challenge infection with *M. oryzae* and *R. solani*. Co-application of 50 μM sodium ascorbate and 100 nM pyocyanin attenuated the pyocyanin-triggered resistance to *M. oryzae*. Similarly, addition of 50 μM ascorbate to the pyocyanin feeding solution alleviated the stimulation of *R. solani* infection by pyocyanin (De Vleesschauwer et al. 2006). Thus, the cumulative results suggest that the differential beneficial effect of pyocyanin in 7NSK2-mediated ISR to *M. oryzae* and *R. solani* is due to its capacity to generate H_2O_2 *in planta*.

2.4 Cell Biology of Pyocyanin-Induced Resistance to M. oryzae

Cytological comparision of the *M. oryzae* infection process and associated host cellular reactions between control and pyocyanin-induced plants revealed that pyocyanin primes rice for potentiated expression of hypersensitive response (HR)-like cell death in response to fungal infection. In control plants, fungal hyphae grew vigorously within penetrated epidermal cells and neighbouring cells by 36 h after infection (Fig. 4A), while in pyocyanin-treated plants fungal ingress was abrogated in the initially penetrated cell as a result of the establishment of HR-like cell death which is characterized by granulation of the cytoplasm and bright autofluorescence

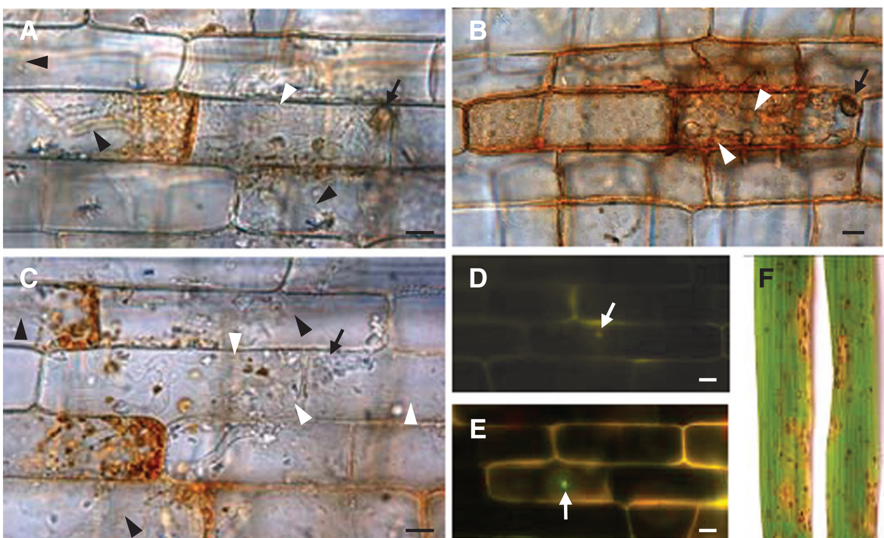


Fig. 4 Interaction phenotypes of pyocyanin-mediated cytological responses of Oryza sativa line CO-39 to Magnaporthe oryzae. (**A–F**) Infection sites inoculated with *M. oryzae*. Scale bars represent 10 μm. (**A**) Vigorous invasion of living tissues in control plants by 36 hpi. Black arrow points to fungal appressorium. (**B**) Expression of HR-like cell death blocks *M. oryzae* in hydroponically grown rice plants amended with 100 nM pyocyanin (36 hpi). (**C**) Addition of 50 μM ascorbate to the pyocyanin feeding solution abrogates pyocyanin-induced HR-like cell death. Arrow points to the site of fungal penetration. (**D**) Faint autofluorescent halo surrounding point of penetration (*white arrow*) as well as weak local autofluorescence in control plants under blue light excitation (24 hpi). (**E**) Epifluorescence image of epidermal cells of pyocyanin-feeded plants responding to *M. oryzae* infection (24 hpi). White arrow indicates position of the fungal appressorium. (**F**) Symptoms of *M. oryzae* on the fourth leaf of control plants (*left*, 7 days post inoculation [dpi]) and pyocyanin-treated plants (*right*, 7 dpi). (Copyright © by the American Phytopathological Society; reprinted from De Vleesschauwer et al. 2006)

of epidermal cell walls (Fig. 4B,D,E). Addition of ascorbate to the pyocyanin feeding solution attenuated the establishment of HR-like cell death (Fig. 4C), suggesting that pyocyanin-derived H_2O_2 orchestrates the priming for HR-like cell death in ISR-expressing tissue. In line with the proposed role for H_2O_2 generation in systemic signaling leading to the establishment of SAR (Alvarez et al. 1998), we speculate that pyocyanin-generated H_2O_2 microburst might likewise function in 7NSK2-mediated ISR by low-level activation of defense responses throughout the plant, thereby contributing to the ISR-induced state.

In summary, the dual role of the phenazine antibiotic pyocyanin in *P. aeruginosa* 7NSK2-mediated ISR suggests that rice requires distinct mechanisms for defense against *M. oryzae* and *R. solani*. On one hand, root treatment with pyocyanin was effective against *M. oryzae*, triggering reiterative H_2O_2 microbursts, and causing rapid HR-associated cell death in response to fungal infection, which most likely leads to breakdown of the biotrophic phase of the *M. oryzae* infection cycle. On the other hand, treatment with pyocyanin significantly promoted subsequent infection

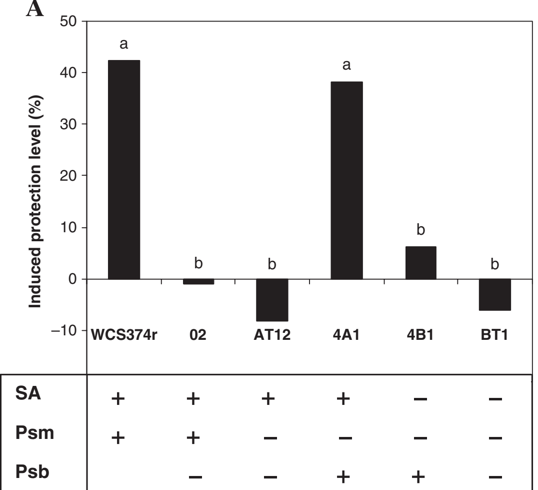
by the necrotrophic pathogen *R. solani* by facilitating pathogen-triggered host cell death. Hence, the oxidative burst and related hypersensitive response (HR) might act as a double-edged sword in the interaction of rice with hemibiotrophic (*M. oryzae*) and necrotrophic (*R. solani*) pathogens. This conclusion is substantiated with recent research by Ahn et al. 2005, demonstrating the differential beneficial effect of the HR as defense mechanism against *M. oryzae* and the necrotrophic rice pathogen *Cochliobolus miyabeanus*.

3 Unraveling *Pseudomonas fluorescens* WCS374r-induced Resistance (De Vleesschauwer et al. 2008)

The gram-negative bacterium *Pseudomonas fluorescens* WCS374r has previously been shown to suppress Fusarium wilt of radish (*Fusarium oxysporum* f. sp. *raphani*) and reduce disease caused by *Ralstonia solanacearum* in *Eucalyptus* (Leeman et al. 1996; Ran et al. 2005). Remarkably, high inoculum densities of WCS374r cultivated at 28 °C failed to elicit ISR in *Arabidopsis* against *P. syringae* cv. *tomato* (VanWees et al. 1997) whereas low inoculum densities or inoculum cultivated at elevated temperatures triggered protection against several microbial pathogens (Djavaheri 2007; Ran et al. 2005). To date, multiple bacterial determinants such as salicylic acid, the siderophore pseudobactin, outer membrane lipopolysaccharides, and additional so far unidentified iron-regulated compounds have been reported to play a crucial role in WCS374r-elicited ISR in dicots (Djavaheri 2007; Leeman et al. 1996, 1995; Ran et al. 2005).

3.1 Involvement of Bacterial Pseudobactin in the Elicitation of ISR by WCS374r

To study the bacterial traits operative in triggering ISR to *M. oryzae*, we screened a series of bacterial mutants deficient in the production of biocontrol-related metabolites for their ability to mount ISR. Pseudobactin was identified as a crucial determinant of WCS374r-triggered ISR. Pseudobactin-negative mutants no longer induced resistance to *M. oryzae*, while the pseudomonine-deficient mutant 4A1, which still produces pseudobactin, was as effective as the parental strain (Fig. 5A). Interestingly, no statistically significant differences in disease severity could be observed between control plants and plants colonized by the pseudobactin-positive but SA-deficient mutant 4B1, suggesting that both pseudobactin and SA are required to trigger ISR. However, this speculation could not be substantiated with feeding experiments using isolated compounds in that purified pseudobactin alone was highly effective in triggering resistance to *M. oryzae*, whereas addition of salicylic acid partially attenuated pseudobactin-induced resistance (Fig. 5B). Taken together, these data indicate that pseudobactin plays a pivotal role in WCS374r-mediated ISR and suggest that salicylic acid negatively interacts with pseudobactin in the elicitation of systemic resistance to *M. oryzae*.



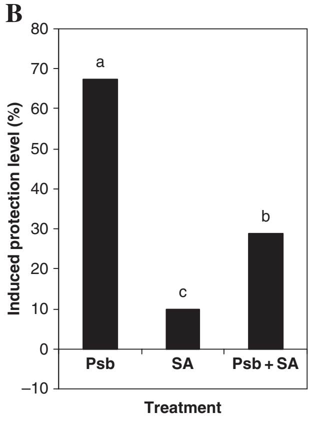


Fig. 5 Role of pseudobactin and salicylic acid in WCS374r-mediated ISR to *M. oryzae*. A, Induced resistance by the wild-type strain WCS374r and its mutants. SA=salicylic acid; Psm=pseudomonine; Psb=pseudobactin. Bars marked with "b" are not different from the control treatment. B, Effect of purified compounds on rice blast severity in a gnotobiotic system. Purified pseudobactin (70 μg/plant) and salicylic acid (1 nM) were added alone or in combination to half-strength Hoagland nutrient solution; 3 days later, plants were challenge inoculated with *M. oryzae*. Different letters indicate statistically significant differences between treatments according to Kruskal-Wallis followed by Mann-Whitney comparison tests ($P = 0.05$). (Copyright © by the American Society of Plant Biologists; adapted from De Vleesschauwer et al. 2008)

3.2 Cell Biology of Pseudobactin-Triggered Resistance to M. oryzae

To further decipher the role of pseudobactin in WCS374r-triggered ISR to *M. oryzae*, we assayed control and pseudobactin-induced plants for several biochemical reactions including accumulation of autofluorescent compounds, callose deposition, lignification, cell wall protein cross-linking, and H_2O_2 generation at various time-points after challenge inoculation. In control plants, fungal hyphae successfully colonized the first-invaded epidermal cell and tended to spread into neighbouring epidermal and mesophyl cells without marked host cellular responses by 38 hpi (Fig. 6A). By contrast, in pseudobactin pre-treated plants, fungal ingress was often arrested in the initially penetrated cell either as a result of invading hyphae-embedding tubules (Fig. 6B) or through a specific cellular reaction type characterized by the occurrence of tubular and round vesicles in the cytoplasm and associated browning of the epidermal cell walls (Fig. 6C). Microscopic analysis of sheath cells under blue light revealed that pseudobactin sensitizes rice seedlings for potentiated deposition of autofluorescent compounds (likely lignin) at sites of potential pathogen penetration (Fig. 6D). Conceivably, enrichment of the host cell walls with phenolics is likely to contribute to the elaboration of permeability barriers

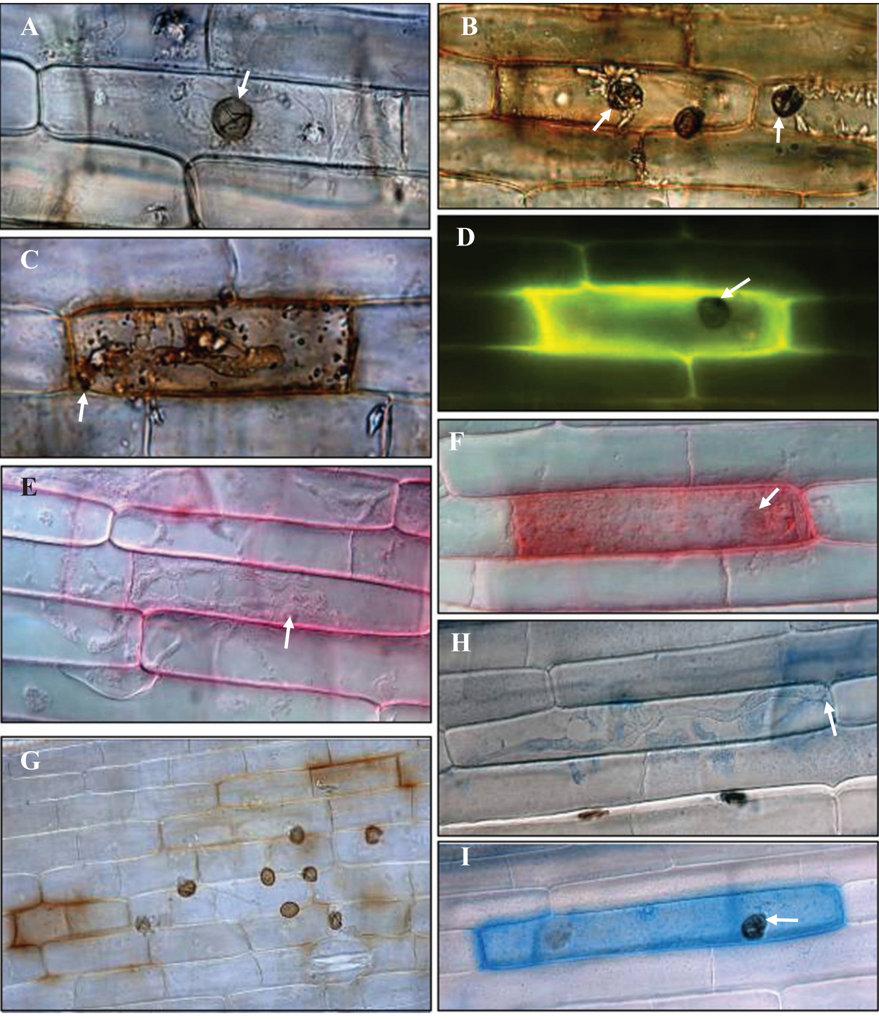


Fig. 6 Microscopic analysis of pseudobactin-triggered resistance to *M. oryzae*. Black arrows point to appressoria or initial points of penetration. (Copyright © by the American Society of Plant Biologists; reprinted from De Vleesschauwer et al. 2008)

preventing pathogen spread and enzymatic degradation. Several studies have convincingly shown that phenolic structures can confer strong rigidity to the host cell walls through peroxidase-mediated cross-linking with pre-existing wall carbohydrates such as hemicellulose, pectin and cellulose. Examination of safranin-stained sheath cells demonstrated that, in pseudobactin-treated plants, abundant cell wall modifications are closely associated with fungal arrest in the first-invaded cell (Fig. 6F), while in control plants, safranin staining was weak and present only in limited zones of the anticlinal cell walls of colonized cells (Fig. 6E). Staining leaf sheath cells with DAB for the presence of H_2O_2 revealed that pseudobactin

triggers a timely and localized oxidative burst in response to fungal infection as early as 24 hpi (Fig. 6G). In control plants, H_2O_2 generation could not be detected prior to 36 hpi. Because protein cross-linking is a potential side effect of H_2O_2 generation that could provide cell-wall reinforcement, we used Coomassie blue staining to visualise protein cross-linking. Whereas no Coomassie blue staining was visible in control plants prior to 48 hpi (Fig. 6H), pseudobactin-induced plants displayed extensive protein cross-linking in both anticlinal and periclinal epidermal cell walls from 36 hpi (Fig. 6I). Summarized, these data indicate that protection afforded by pseudobactin pre-treatment involved priming for potentiated host cell wall rigidification upon challenge infection with *M. oryzae*.

3.3 An SA-Independent Signaling Pathway Controls WCS374r-mediated ISR to M. oryzae

In an attempt to unravel the signal transduction pathway(s) underlying WCS374r-triggered ISR in rice, bioassays were performed with transgenic and mutant rice lines impaired in various structural components of known signaling routes. Figure 7 shows that transgenic SA non-accumulating NahG rice plants and wild-type Nipponbare plants are equally responsive to WCS374r-mediated ISR, suggesting that WCS374r triggers ISR in rice either by activating the SA signaling cascade downstream of SA accumulation or by functioning independently of SA. Conversely, ISR induced by WCS374r was blocked in the ethylene (ET)-insensitive *ein2a* transgenic line and the JA-deficient *hebiba* mutant line, indicating that signal transduction leading to WCS374r-mediated ISR requires both an intact response to ethylene and a functional jasmonate pathway (Fig. 7). Hence, WCS374r appears to trigger an

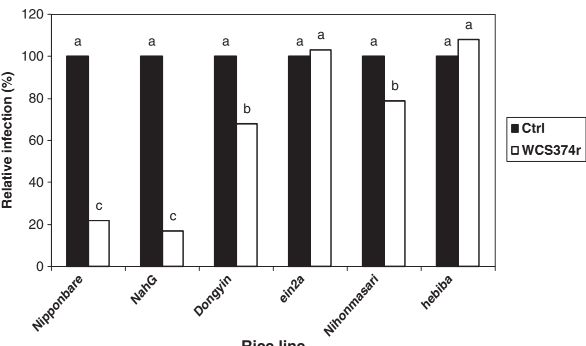


Fig. 7 Quantification of WCS374r-mediated ISR to *M. oryzae* in various rice lines. NahG = SA non-accumulating Nipponbare transgenic line; *ein2a* = OsEIN2a antisense line made in Dongyin background, ethylene-insensitive; *hebiba* = JA-deficient mutant line of cv. Nihonmasari. Different letters indicate statistically significant differences between treatments (Kruskal-Wallis followed by Mann-Whitney non-parametric tests, P = 0.05). (Copyright © by the American Society of Plant Biologists; reprinted from De Vleesschauwer et al. 2008)

SA-independent but ET-, JA-regulated signaling cascade, which resembles the model signal transduction pathway controlling WCS417r-mediated ISR in Arabidopsis.

To further probe whether WCS374r elicits an SA-independent signaling route, we analyzed the expression patterns of the SA- and BTH-responsive PR genes *PR1b* and *PBZ1* in control and pseudobactin-induced plants by quantitative RT-PCR. In line with previous reports (Agrawal et al. 2001), *PR1b* transcript levels responded strongly to infection with *M. oryzae* in control plants. However, the pathogen-induced up-regulation of *PR1b* was significantly blocked in pseudobactin-treated plants (Fig. 8A). Transcript accumulation of the *PBZ1* gene mirrored the profile observed for *PR1b* (Fig. 8B). Although the differential expression of *PR1b* and *PBZ1* in pseudobactin-induced plants compared to control plants is most likely the consequence rather than the cause of pseudobactin-increased blast resistance, these results further support the notion that ISR triggered by pseudobactin from WCS374r follows an SA-independent signaling cascade. In view of the reported antagonistic interplay between different signaling pathways, the SA-independency of pseudobactin- and WCS374r-mediated blast resistance might also explain why exogenous SA inhibited the elicitation of ISR by pseudobactin.

3.4 Is the ISR-Triggering Capacity of Pseudobactin Related to its Ability to Cause Intracellular Iron Depletion?

Pseudobactins (also called pyoverdines) are defined as low molecular weight Fe^{3+} specific ligands that are produced by fluorescent pseudomonads in response to cellular iron deficiency (Lemanceau et al. 2007). Although pseudobactins are all

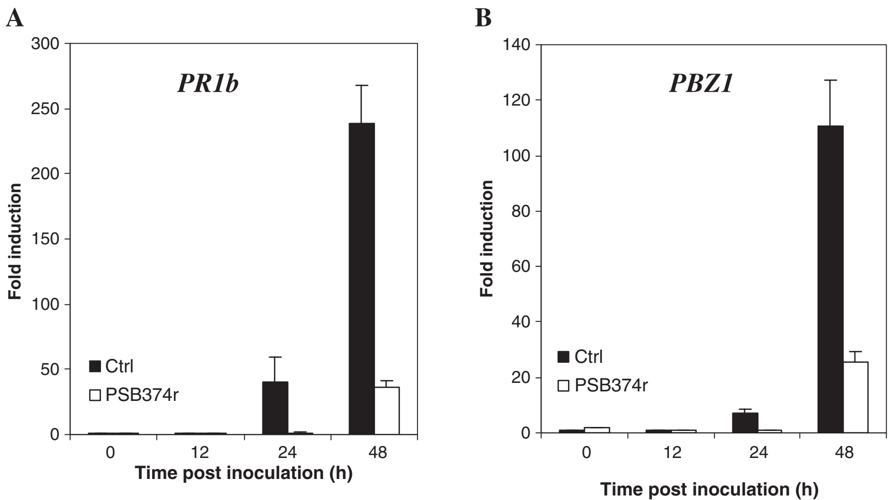


Fig. 8 Quantitative RT-PCR analysis of the SA-responsive PR genes *PR1b* and *PBZ1* in control and pseudobactin-induced plants in response to challenge infection with *M. oryzae*. (Copyright © by the American Society of Plant Biologists; reprinted from De Vleesschauwer et al. 2008)

chromopeptides composed of a quinoleinic chromophore bound to a peptide and an acyl chain (Meyer et al. 1987), fluorescent pseudomonads display a wide structural variety in their pseudobactins (Hofte 1993). This structural heterogeneity begs the question of whether the ISR-triggering potential of PSB374r is conserved among bacterial pseudobactins or rather a strain- and pseudobactin-specific feature. To answer this question, ISR feeding experiments with pseudobactins isolated from diverse biocontrol-associated *Pseudomonas* strains were performed. Interestingly, pseudobactin purified from *P. putida* strain WCS358 was ineffective in triggering resistance to *M. oryzae* while this metabolite is an essential determinant of WCS358-mediated ISR in dicot plants. Furthermore, bacterial mutant analysis and feeding experiments revealed that pyoverdin from *P. aeruginosa* 7NSK2 is unable to significantly reduce blast severity. Thus, only specific pseudobactins are able to increase blast resistance. Leeman and associates (Leeman et al. 1996) obtained similar results when studying the involvement of pseudobactins in *P. fluorescens*-mediated ISR to Fusarium wilt in radish. To date, it is unknown how pseudobactins are perceived by plants. Intriguingly, several lines of evidence suggest that the ISR-triggering potential of a specific pseudobactin is closely associated with its capacity to deprive rice from iron, leading to intracellular iron depletion (data not shown). Consistent with this hypothesis, targeted alterations in iron homeostasis were recently reported to underlie disease resistance responses including H_2O_2 accumulation, cell wall fortification and PR gene expression in several cereals (Liu et al. 2007).

4 Conclusions

Rice blast control in the 21st century faces considerable challenges. In light of the continuing problems of pathogen adaptability leading to breakdown in the effectiveness of genotype-specific resistance conferred by major resistance genes, approaches capitalizing on the plant's own defensive repertoire, such as rhizobacteria-mediated induced systemic resistance, could hold great potential for sustainable rice production in the future. Here, we demonstrate the ability of the PGPRs *P. aeruginosa* 7NSK2 and *P. fluorescens* WCS374r to mount ISR to *M. oryzae*. Despite triggering similar levels of protection against *M. oryzae*, 7NSK2 and WCS374r clearly employ different resistance strategies to trigger ISR. Whereas 7NSK2 is speculated to secrete pyocyanin on the roots, leading to reiterative H_2O_2 microbursts in systemic leaves that sensitize the tissue for potentiated expression of HR-like cell death upon challenge infection, WCS374r-mediated ISR depends on production of the siderophore pseudobactin, a process shown to activate an SA-independent signaling pathway culminating in a pronounced multifaceted cellular defense response upon infection with *M. oryzae*. Moreover, host defense responses operating in WCS374r-mediated ISR show only partial overlap with those observed in BTH-induced resistance or R gene-mediated resistance, suggesting a great degree of flexibility in the molecular processes leading to ISR and blast resistance.

Further elucidation of host defense responses and bacterial traits underpinning ISR to *M. oryzae* will not only advance our fundamental understanding of rhizobacteria-mediated resistance but might also help fine-tune transgenic approaches for enhancing disease resistance in rice and other cereals through effective manipulation of the biochemical pathways leading to systemically activated plant defense. Furthermore, this knowledge could open new opportunities for the use of key ISR target genes in rice breeding programs and should enable a more rapid and reliable development of biological, biochemical or synthetic blast resistance activators in the future.

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Part III
Pathogen Population and Disease Control

World Population Structure and Migration of the Rice Blast Fungus, *Magnaporthe oryzae*

Didier Tharreau, Isabelle Fudal, Dodelys Andriantsimialona, Santoso, Dwinita Utami, Elisabeth Fournier, Marc-Henri Lebrun and Jean-Loup Nottéghem

Abstract The clonal structure of *Magnaporthe oryzae* populations was observed in various countries. This information was used to propose new resistance deployment strategies. However, our understanding of how new virulent races appear and spread remains limited. Population genetic analyses, with neutral and selected markers, provide tools to evaluate such events. We used microsatellite markers to study populations at the worldwide scale. We observed three major genetic groups. Two groups include isolates of only one mating each. The third group includes isolates of both mating types. During natural epidemics spores disperse over short distances (1–5 m). This is likely to cause the partial geographic structuring we observed. However, long distance migrations are possible through the transportation of infected seeds. We observed that, in some cases, one population is more closely related to populations from other continents than from a population from the same area. Long distance migration was also confirmed by studying the distribution of the genotypes of the cloned avirulence gene *ACE1*. Two major virulent genotypes were identified. These genotypes appeared by a complex duplication/deletion event. These two genotypes are widely distributed over different continents. Altogether, these results suggest a unique apparition event followed by long distance migrations of virulent genotypes.

Keywords Avirulence · Mating type · Migration · Population · Sexual reproduction

1 Introduction

The diversity and structure of *Magnaporthe oryzae* populations on rice were described in many countries during the last 20 years using RFLP (Levy et al. 1991; Roumen et al. 1997), rep-PCR markers (George et al. 1998), RAPD (Hong et al. 1996; Ross et al. 1995) or AFLP. Most of these population studies used RFLP

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markers generated by MGR586, a repeated and dispersed sequence related to DNA transposable elements (Chen et al. 1995; Farman et al. 1996; Hamer et al. 1989; Levy et al. 1991, 1993; Roumen et al. 1997; Zeigler et al. 1995). These techniques brought important knowledge on population diversity and structure at the field and country level. The genetic structure of the *M. oryzae* populations pathogenic to rice clearly showed that clonal reproduction is the rule in most rice growing areas (Chen et al. 1995; Levy et al. 1991, 1993; Roumen et al. 1997; Shen et al. 1996; Sone and Tomita 2000; Viji et al. 2000). Despite these clear results, the existence of hermaphroditic strains in some restricted regions of the world leads to the assumption that sexual reproduction could occur in the field in some limited areas. The first studies on fertility of *M. oryzae* showed that hermaphroditic isolates collected on rice were very rare (Kato and Yamaguchi 1982; Yaegashi and Yamada 1986; Notteghem and Silué 1992). Hermaphroditic isolates pathogenic to rice from the Yunnan province of China were identified independently by Hayashi et al. (1997b) and our group (unpublished data). More recently, hermaphroditic isolates were collected in Thailand (Luangsa-Ard et al. 1997; Mekwatanakarn et al. 1999) and India (Kumar et al. 1999) suggesting that sexual recombination could occur in limited areas spread all over the South foothills of the Himalayas. Sexual reproduction between field isolates of the rice blast pathogen was obtained *in vitro* either on culture medium (Silué and Notteghem 1990; Hayashi et al. 1997a; Luangsa-Ard et al. 1997) or dead tissue (Silué and Notteghem 1990; Hayashi et al. 1997a). Despite careful observations, perithecia were never reported in the field (Zeigler 1998; Hayashi et al. 1997a). However, the existence of limited sexual reproduction in restricted areas can not be ruled out. Provided that appropriate molecular markers are used, evidence of recombination can be detected by population genetic analysis.

Population genetic structures were compared to pathotypic structures. The relationship between the genetic structure, deduced from neutral molecular markers, and the pathotypic structure, showed a range of situations varying from a one lineage-one race (USA, Levy et al. 1991; Europe, Roumen et al. 1997; Morocco, El Guilli and Tharreau unpublished) to almost a one genotype-one race correspondence (China, Shen et al. 1996; Colombia, Levy et al. 1993; Côte d'Ivoire, Bouet and Tharreau unpublished; Japan, Sone and Tomita 2000; Philippines, Zeigler et al. 1995). These studies have helped in choosing appropriate isolates for resistance characterization and to propose strategies to breed for durable resistance to blast disease (Zeigler et al. 1994). This background information is the basis to understand rice blast population evolution. But, to date, our understanding of how new virulent races appear and spread is limited. For example, the relative importance of short and long distance migration in the spreading of new virulent races is unknown. However, this information is needed to determine at which scale the deployment of a resistance strategy is likely to be effective and durable. Population genetics can be used to provide information on the importance and scales of migrations.

The development of microsatellite (SSR) markers was shown to be relatively difficult for fungi (Dutech et al. 2007). However, using the *M. oryzae* genome sequence, we recently developed a set of 18 SSR markers for population studies

(Adreit et al. 2007). We used these markers to analyze the diversity and structure of *M. oryzae* populations at the worldwide scale.

2 The Rice Blast Populations are Structured at the Worldwide Scale, and the Himalayan Foothills are Candidate Regions for the Center of Origin

We genotyped more than 1,700 isolates from 40 countries with 13 SSR markers. This sample included reference isolates from previous studies and real populations (one site, one year) collected recently (1999–2006). The world population structure shows evidence for some geographic structuring. For example, genotypes of European isolates are similar. Genotypes of isolates from Madagascar are also similar, but very different from European genotypes and significantly different from genotypes of isolates from Indonesia. This suggests limited migrations between these regions. However, genotypes of isolates from the North and South Americas, and West Africa do not cluster and show similarities with genotypes from other geographic origins. This result suggests intercontinental migrations.

A structure in three major genetic groups was observed (Fig. 1). One group corresponds to isolates of mating type *Mat1.1*. The second group gathers isolates of *Mat1.2*. The third group contains isolates of both mating types. Diversity is higher in Asia, especially in the countries of the Himalayan foothills. This area is the

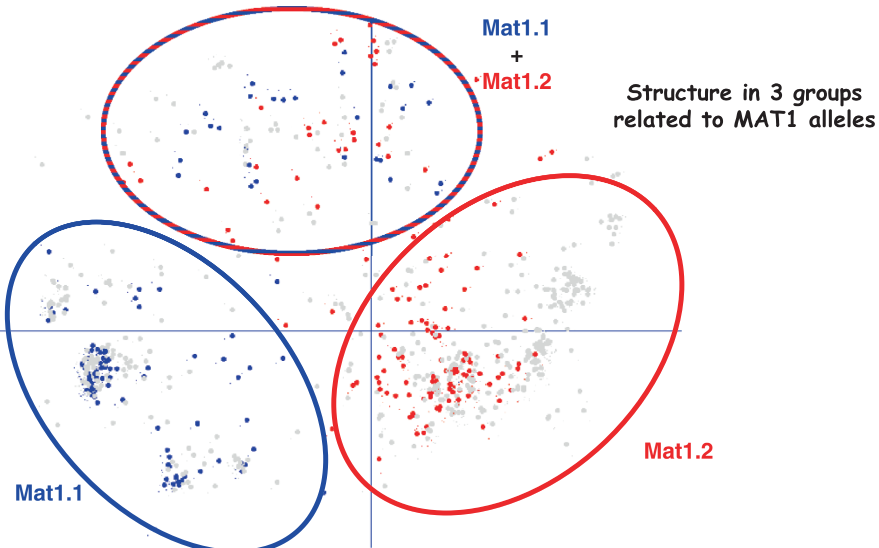

Fig. 1 *Magnaporthe oryzae* world population structure. More than 1,700 isolates from 40 countries were genotyped with 13 microsatellite markers. In this principal component analysis graph, each point represents a different isolate

only one where female fertile isolates (able to produce perithecia and ascospores *in vitro*) were identified. Both mating types are also present in these populations. The observed structure is consistent with the hypothesis of a center of origin in the Himalayan foothills where sexual reproduction was taking (or still takes) place. We hypothesize that *Mat1.1* and *Mat1.2* groups have arisen by migration and loss of sexual reproduction, following founder effects.

3 Short or Long Distance Migration? Both

World population structure clearly shows evidence for intercontinental migrations. But, previous work showed that spore dispersal is limited. So, structuring at a local scale is expected. We genotyped five populations from the Central part of Madagascar and three from the Camargue area in France. Data analyses showed that populations can be differentiated at a local scale (10–20 km or less). These results suggest limited migration between geographically close populations.

Our apparently contradictory results from studies at two different geographic scales are explained by two distinct modes of dissemination. The structure on a local scale is consistent with short distance spore dispersal (1–5 m) observed during natural epidemics. Long distance migrations, including intercontinental, are possible through the transport of infected materials (probably seeds).

4 Intercontinental Migration Can Play a Role in Spreading Virulent Strains: The *ACE1* Story

The world population structure supports the hypothesis of intercontinental migrations. But, what is the potential impact of these migrations on spreading virulent strains? The avirulence gene *ACE1* was cloned by map based cloning (Dioh et al. 2000; Böhnert et al. 2004). It encodes a Polyketide Synthase coupled to a Non Ribosomal Peptide Synthase (PKS-NRPS; Böhnert et al. 2004). These enzymes contribute to the biosynthesis of secondary metabolites. We showed that, in a virulent mutant obtained during crossings, the gene was interrupted by retrotransposon (Fudal et al. 2005). But, we were interested in studying the diversity of *ACE1* alleles in field populations of the blast fungus. We first determined by RFLP analysis the different possible genotypes of the cloned avirulence gene *ACE1* in a collection of very diverse avirulent and virulent isolates. Only one genotype was identified for avirulent isolates. Two major genotypes were identified for virulent strains. These genotypes appeared by a complex duplication/deletion event of *ACE1*. Then, we designed a PCR test Couch et al. 2005 to identify these three genotypes and studied their distribution at the worldwide scale. We determined the *ACE1* genotype of more than 800 isolates. Avirulent isolates were the most frequent, and were sampled all over the World (Fig. 2). The frequencies of virulent genotypes varied with geographic origin. The two major virulent genotypes were widely distributed over

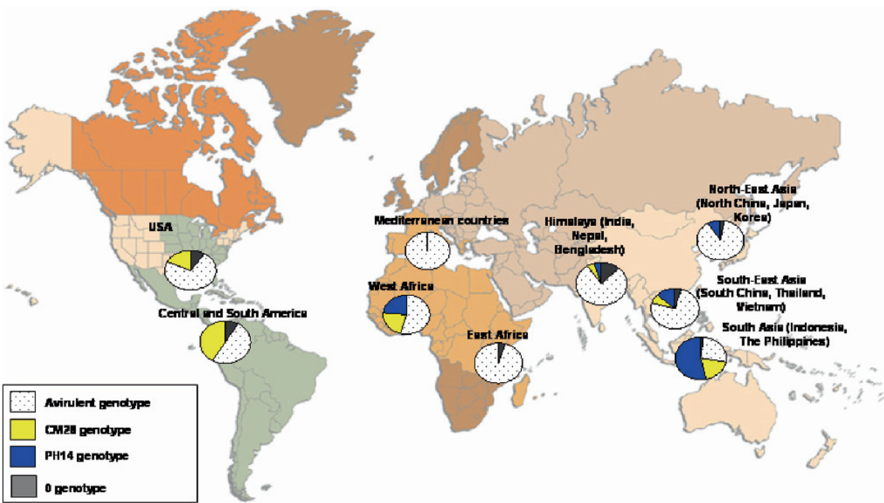

Fig. 2 Geographic distribution of *ACE1* genotypes. The *ACE1* genotype of 800 isolates was determined by PCR. CM28 and PH14 genotypes are virulent genotypes. The “0” genotype corresponds to isolates showing no amplification

different continents (Fig. 2). Because these two genotypes originate from a complex event, we believe that this event occurred only once. Thus, the wide geographic distribution of virulent isolates results from intercontinental migrations that were probably favored by the exchange of infected seeds during the Green Revolution. *ACE1* virulent isolates overcome the resistance gene *Pi33* (Berruyer et al. 2003), a gene relatively frequent in improved semi-dwarf varieties of the Green Revolution, including the most widely grown variety IR64 (Ballini et al. 2007). At that time, semi-dwarf varieties have been exchanged to create improved materials, and these varieties probably served as a vector for virulent strains of the blast fungus.

5 Conclusions and Prospects

During the past 20 years, population studies of the rice blast fungus were mainly carried out at the country scale. We studied the population structure at the world-wide scale. This study showed that the Himalayan foothills are a center of diversity of *M. oryzae*. We hypothesize that this area could be the Center of origin of the rice blast fungus. We also hypothesize that sexual reproduction was taking place in this area, but was lost in other parts of the World following migration and founder effects. Whether sexual reproduction is still occurring in some limited areas remains to be determined.

Intercontinental migrations were established by two different approaches. The distribution of virulent genotypes of the *ACE1* avirulence gene is consistent with migrations between continents. Population structure at the worldwide scale shows that geographically distant populations are genetically related. North and South

American and West African populations of the blast fungus probably resulted from several introduction events. How many migration events can we trace? When did they take place? What were the migration routes? We will search for answers to these questions to provide useful information to improve the future strategies of resistance gene deployment.

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Examination of the Rice Blast Pathogen Population Diversity in Arkansas, USA – Stable or Unstable?

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Abstract Over the past 17 years, isolates of *Pyricularia oryzae* (= *P. grisea*) have been recovered from commercial rice fields in Arkansas. Annual samples have typically included 100–500 isolates recovered from 5 to 10 cultivars from 10 different counties with the majority of the isolates being recovered from neck blast samples. Isolates of *P. oryzae* were characterized using a number of tests including DNA fingerprinting with MGR586, mitochondrial DNA RFLPs, mating type, vegetative compatibility, and virulence. Although up to eight different MGR586 DNA fingerprint groups (A-H) have been identified among contemporary and archived isolates of *P. oryzae* in the U.S., only 4 MGR586 DNA fingerprint groups (groups A, B, C, and D) have been identified since monitoring the populations in Arkansas beginning in 1991. There is a complete correspondence between the four MGR586 DNA fingerprint groups (A-D) and the four distinct genetic vegetative compatibility groups (VCGs 01-04). Furthermore, all isolates belong to a single mtDNA RFLP haplotype and all isolates within a given group are of a single mating type. In addition, some yearly samples have even shown that a single haplotype often makes up the majority of the isolates within a given fingerprint group or VCG. For example, over 60% of the isolates recovered in a given season belonged to 1 of 4 distinct clones. Thus, it is evident that the rice blast pathogen population in Arkansas has remained stable over the past 17 years with regard to these four MGR586 DNA fingerprint groups. Although all 4 MGR586 groups can typically be found in the annual samples of the contemporary population, there appears to be a strong bias for group A isolates in more recent samplings (since 2000). Over 80% of the isolates recovered between 2000 and 2006 were in MGR586 group A, belonged to VCG US001, had a single mtDNA RFLP haplotype, and were a single mating type (*mat1-1*). The data indicate that the population is strongly influenced by host genotype. Evaluation of virulence indicates that isolates within a group are clearly more similar within a group than between groups; however, there is some virulence diversity within each of the genetic groups identified. In addition, a distinct "shift" in virulence among field isolates to overcome the *Pi-ta* resistance gene has occurred among MGR586

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group B isolates. The immergence of this “race-shift” has occurred among field isolates in MGR586 group B and also can be generated experimentally in greenhouse selections among isolates in group B; this race shift is associated with changes in *AVR-Pita*.

Keywords Field isolates · Lineage analysis · Fingerprinting · Resistance durability

1 Introduction

Pyricularia oryzae (teleomorph, *Magnaporthe oryzae*) is an aggressive fungal pathogen that causes rice blast disease and is a one of the main limiting factors for high yielding rice production throughout the world (Bonman, 1992; Marchetti et al., 1976; Ou, 1985).The genus *Magnaporthe* collectively parasitizes more than 50 hosts but individual isolates often have more limited host range and cross-infectivity (Couch et al., 2005; Valent and Chumley, 1991; Zeigler, 1998). *P. grisea/P. oryzae* has been reported on many host families including *Musaceae*, *Zingiberaceae*, *Cyperaceae* and *Commelinaceae*, as well as Gramineae (Lavanya and Gnanamanickam, 2000) but P. oryzae has been proposed as the species infecting rice (Couch and Kohn, 2002; Rossman et al., 1990).

Blast is considered one of the main diseases of rice because of its worldwide distribution and destructiveness under favorable conditions. Rice blast has been reported in nearly every rice production region in the world (Bonman, 1992; Ou, 1985). In the United States, rice blast occurs in all of the southern rice-producing states of Arkansas, Texas, Louisiana, Mississippi, Missouri, Florida (Lee, 1994; Marchetti et al., 1976) and more recently in California (Greer and Webster, 2001).

Intensive studies on virulence diversity of *P. oryzae* began in the 1950s when some rice cultivars with resistance to blast suddenly became very susceptible to the rice blast pathogen (Ou, 1980a).The high variability in pathogenicity and virulence among rice blast isolates continues to present challenges to plant breeders to develop rice cultivars with durable resistance to the rice blast pathogen. In many irrigated lowland rice producing areas, host resistance has been widely used to control rice blast; however the frequent emergence of new pathogenic races continues to pose a threat to stable rice production (Leung and Taga, 1988). In southern states of the U.S., ‘Katy’, a *Pi-ta* containing cultivar, has been widely used for controlling rice blast since its release in 1989 (Lee et al., 2006; Moldenhauer et al., 1990). However, the recent emergence of isolates that can overcome *Pi-ta* mediated resistance in the southern U.S. germplasm presents a major concern (Lee et al., 2009).

Molecular markers have been a useful way to characterize population diversity of many plant pathogenic fungi. Populations of the rice blast pathogen from throughout the world have been studied for their phenotypic and genotypic variation (Correll and Gordon, 1999; Zeigler, 1998). Hamer et al. (1989) identified a family of dispersed repetitive DNA sequences within the genome of *P. oryzae*, and these repetitive elements have become useful tools for examining genetic diversity

within and between populations of the rice blast pathogen. Levy et al. (1991) initially examined the relationship between virulence and distinct genetic groups or lineages. In previous years, eight MGR586 genetic groups (designated A through H) have been identified in the United States (Levy et al., 1991; Correll et al., 2000a; 2000b; 2000c; Xia et al., 1993, 2000). Arkansas is the largest rice producing state in the U.S. with annual production occurring on approximately 700,000 ha representing about 45% of the total U.S. production (http://www.nass.usda.gov/QuickStats/). The eight MGR586 fingerprint groups identified in the U.S. have been found among both archived and contemporary isolates of the rice blast pathogen, but only four groups (A, B, C, and D) have been recovered since 1991 (Correll et al., 2000). The objective of the current effort was to provide an overview of the rice blast pathogen population diversity sampled in Arkansas over the past 17 years.

2 Materials and Methods

2.1 Collection of Isolates

Over 3000 isolates were collected in the U.S. with the majority of the isolates recovered from commercial rice fields in Arkansas between 1991 and 2006 (Table 1). Isolates were recovered from rice leaf tissue or panicles with symptoms of rice blast.

Table 1 Frequency of MGR586 groups and/or VCGs identified among isolates of *Pyricularia oryzae* sampled in Arkansas between 1991 and 2006[1]

Year	Total No. of isolates examined	Percentage of isolates in each MGR586 Group or VCG			
		Group A/VCG 01	Group B/VCG 02	Group C/VCG 03	Group D/VCG 04
1991	113	63.7	9.7	15.9	10.6
1992	470	30.0	33.4	24.0	12.6
1994[2]	424	43.9	49.3	1.2	5.7
1995[2]	94	25.5	74.5	0.0	0.0
1992–1996[3]	70	40.0	25.7	21.4	12.9
2000	357	96.1	2.7	0.0	1.2
2001	92	96.7	3.2	0.0	0.0
2002	276	94.4	5.2	0.0	0.4
2003	342	96.8	2.4	0.0	0.6
2004[4]	498	78.5	16.4	0.0	0.2
2005[4]	88	61.1	38.9	0.0	0.0
2006	185	94.9	5.1	0.0	0.0
Total	3, 009				

[1] Isolates were typically recovered from symptomatic rice from commercial rice fields in Arkansas unless otherwise noted.
[2] Samples were collected from three experimental plots whereby 20 cultivars or breeding lines were grown within a commercial rice field (Ross, 1997).
[3] A collection of isolates representing a wide range of races from AR, TX, LA, MS, MO, and FL were examined (Correll et al., 2000).
[4] The frequencies were biased due to more intensive sampling during epidemics on the *Pi-ta* containing cultivar Banks.

Typically, approximately 5 plants were sampled per location in a field from an area of $5 \times 8\,m^2$ and each location within a field was 35 m apart. No more than one isolate was recovered and stored from a single plant. For recovery, conidia from the lesion surface were spread onto 2% water agar with a sterile loop and incubated overnight. Single germinating conidia were isolated and transferred to water agar and incubated for 5–6 days. A single monoconidia isolate from a given sample was transferred to a filter paper on rice bran-agar (RBA) medium and stored as previously described (Correll et al., 1986, 2000; Leung and Taga, 1988).

2.2 MGR586 Fingerprints, mtDNA RFLPs, and Vegetative Compatibility Tests

Standard MGR586 DNA fingerprinting and mtDNA RFLPs were determined as previously described (Correll et al., 2000a; 2000b; 2000c). Four reference isolates A598, A264, A119, and A347 representing one of the four MGR586-delineated lineages A, B, C, and D, respectively, were used for MGR586 group comparisons. MGR586 lineages were assigned based on similarity in DNA fingerprints when genomic DNA was restricted with *Eco*RI as previously described (Correll et al., 2000). Vegetative compatibility tests were conducted using nitrate non-utilizing mutants as previously described (Correll et al., 2000a; 2000b; 2000c).

2.3 Virulence Tests

A primary differential set of 25–30 cultivars was used to characterize isolates for virulence diversity under greenhouse conditions (Table 2). Representative isolates from the various MGR586 DNA fingerprint groups where subsequently characterized for virulence diversity on additional cultivars. Greenhouse inoculation tests were conducted by growing plants to the 3–4 leaf-stage (approximately 2–3 weeks) prior to inoculation. Conidia were collected from 8- to 10-day-old cultures grown on RBA by washing the agar surface with sterile water. The concentration of conidia was adjusted to 2×10^5 conidia per milliliter. Each tray was sprayed with 50–70 ml of inoculum with a compressed-air sprayer. Plants were incubated in a dew chamber at 100% relative humidity (RH) at approximately 21–22 °C for 24 h. Plants were then placed back into the greenhouse at approximately 28–30 °C and scored for disease symptoms. Controls included plants sprayed with water.

Plants were evaluated for disease reaction 7 days after inoculation using a 0–9 scale (IRRI evaluation system and Xia et al., 1993) where 0 = no infection observed; 1 = small brown specks, pinpoint infections small < 1 mm; 2 = small rounded infections with open centers, lesion small < 2 mm; 3 = small infections with open centers beginning to expand but small < 3 mm; 4 = typical susceptible blast lesions with expanding open centers > 3 mm on $< 10\%$ of the leaf area evaluated; 5 = susceptible type lesions with expanding open centers on 10–25% of the leaf area evaluated; 6 = susceptible type lesions with expanding open centers on 26–50% of

Table 2 Disease reactions among representative isolates within MGR586 groups A, B and D on a primary set of rice differentials based on greenhouse inoculations

Cultivars	Virulence reaction[1]		
	MGR586 Group A (VCG US-01)	MGR586 Group B (VCG US-02)	MGR586 Group D (VCG US-04)
XL-6[2]	R2	S3	R/S4
Earl	R/S	R/S	S
LaGrue	S	S	S
Bengal	S	R	S
Wells	S	S	S
Cocodrie	R/S	R	R/S
Priscilla	R/S	R/S	R/S
AB6564	S	R/S	S
Arhent	R	R	R/S
Drew	R	R	R
RU9801148	S	R/S	S
AB1542	S	R/S	S
Jefferson	S	R	R/S
AB3004	R/S	R/S	R/S
Kaybonnet	R	R	R
AB6565	S	R/S	S
Cypress	S	R/S	S
M-201	S	S	S
RU9601099	S	R	S
Madison	R	R	R
LeMont	S	S	S
Francis	S	S	S
Usen	S	R	S
STT	S	S	S
NP-125	R/S	R/S	S
Mars	S	R	S
Starbonnet	S	S	S
Zenith	S	R	R/S
Dular	S	S	S
OL-5	R	R	R

[1] Virulence reactions were characterized as R = resistant (mean disease rating < 3.5); S = susceptible (mean disease rating > 4.5); and R/S indicates that some isolates yielded R reactions whereas others an S reaction.
[2] Cultivars highlighted in gray can be used to distinguish group A, B, and D isolates.

the leaf area evaluated; 7 = susceptible type lesions with expanding open centers on 51–75% of the leaf area evaluated; 8 = susceptible type lesions with expanding open centers on 76–90% of the leaf area evaluated; 9 = susceptible type lesions with expanding open centers on $> 90\%$ of the leaf area evaluated. A mean reaction of 0–3.5 was considered resistant, 3.5–4.5 intermediate and ≥ 4.6 susceptible.

The differential cultivars were grouped based on their disease reaction (either resistance with a disease rating of 0–3 or susceptible with a disease rating of 4–9). A matrix table was generated and simple matching (SM) coefficient of similarity were calculated with the formula m/n where m = the number of matches shared

by a given pair of isolates, and n = the total number of sample size for that pair of cultivars. Cluster analysis was performed on the similarity coefficients by the unweighted paired-group method, arithmetic mean UPGMA procedure using NTSYSpc software version 2.11W (Exeter Software, Setauket, NY). Dendrograms were generated by the following procedure in NTSYSpc: Similarity SimQual – Coefficient SM, SAHN using clustering method UPGMA, and TreeView version 1.6.5. Bootstrap analysis of the data was conducted with 1,000 replications using Winboot software to test reliability and robustness of the dendrograms produced (www.irri.org/science/software/winboot.asp).

2.4 Race Nomenclature

The nomenclature used to characterize virulence diversity within populations of the rice blast pathogen has always been problematic. In a seminal paper to try to standardize the race nomenclature, Ling and Ou (1969) proposed the use of eight differential cultivars (A – Raminad Str. 3; B – Zenith; C – NP125; D – Usen; E – Dular; F – Kanto 51; G – Sha-tiao-tsao; and H – Caloro). Each of these eight differentials (A-H) were also assigned a numerical value of 128, 64, 32, 16, 8, 4, 2, and 1, respectively. Isolates were then assigned an international "I" designation based on the differentials that were susceptible. For example, if Zenith was the first differential in the ordered set that was susceptible, it was assigned to group B or IB. Then a numerical value was assigned to the IB by adding up the numerical values of the subsequent differentials that were resistant and adding 1. For example, for race IB49, where the reactions on the eight ordered differentials are RSRRSSSS, Zenith is the first differential susceptible for the IB and NP125 (value 32) and Usen (value 16) are resistant. Thus $32 + 16 + 1 = 49$ for the designation IB49.

Although this system of nomenclature is rather elegant, it is not very functional other than to describe broad virulence groups. Typically, for any particular geographical area, there are a number of local cultivars that can clearly subdivide any particular international race into various virulence phenotypes.

3 Results and Discussion

Samples of the rice blast pathogen population over the past 17 years in Arkansas indicated a relatively consistent persistence of the predominant MGR586 fingerprint groups and/or VCGs. Although eight groups have been identified in the U.S. (Correll et al., 2000a; 2000b; 2000c; Levy et al., 1991; Xia et al., 1993), only four groups (Groups A, B, C, and D) appear to persist in the more contemporary population (Table 1 and Fig. 1). Moreover, there is a complete correspondence between the MGR586 group, the VCG, and the mating type among the isolates that have been examined (Correll et al., 2000). Interestingly, intensive sampling since 2000 has indicated that the vast majority of isolates recovered belong to group A (Table 1). In fact, the group A isolates represented over 90% of the isolates sampled except

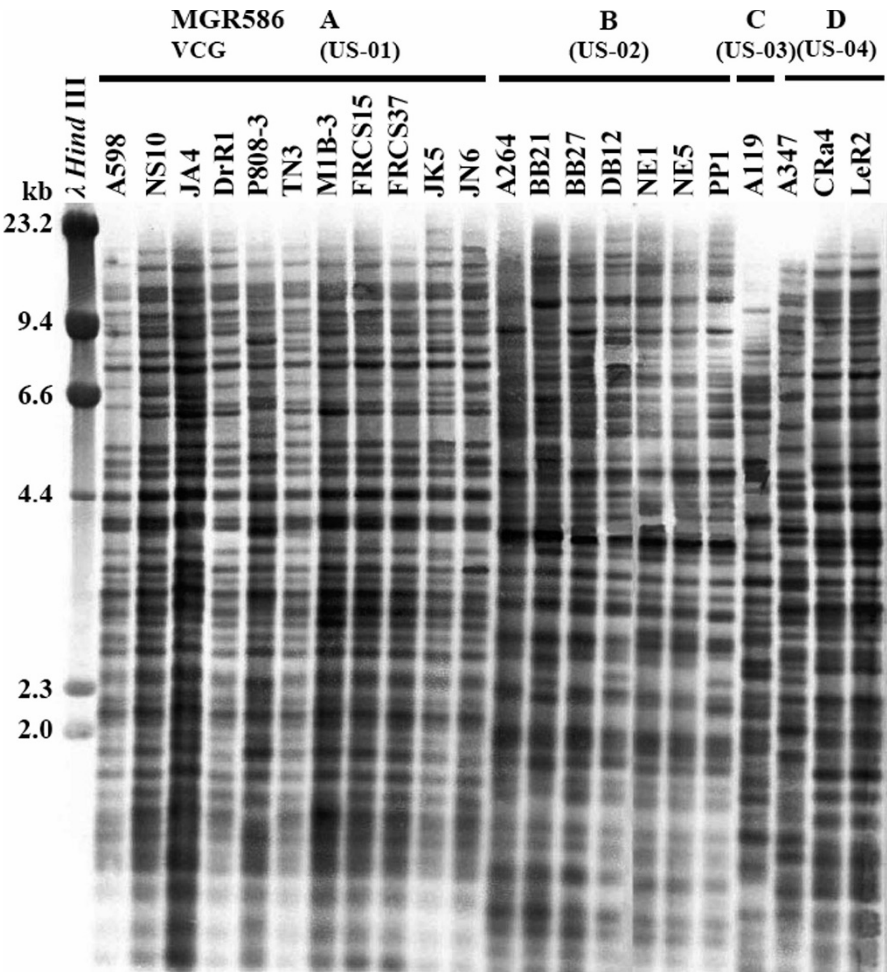

Fig. 1 Genomic DNA of isolates of *Pyricularia oryzae* restricted with *Eco*RI and probed with a chemilluminescent labeled MGR586 probe. The four MGR586 groups (A–D) and the four vegetative compatibility groups (VCGs 01–04) are represented

in 2004 and 2005 when an effort was made to collect isolates that were virulent on Banks which were known to belong to group B (Boza, 2005). Although group B isolates that could attach *Pi-ta* containing cultivars were identified as early as 1994 from field isolates (Correll et al., 2000a), Banks was the first commercial cultivar that contained Pi-ta on which epidemics occurred which resulted in an economic impact (Lee et al., 2005a,b). The field isolates which were identified that could overcome Pi-ta also showed to have an alteration in AVR-Pita (Boza, 2005; Jia et al., 2004; Zhou et al., 2007).

Table 3 Five rice cultivars from a modified differential set distinguished seven virulence phenotypes within a subset of 40 rice blast isolates from MGR586 fingerprint group A collected in Arkansas

MGR586 Group A / VCG US-01 Isolates	Cultivars				
	Coccodrie	Priscilla	Jefferson	Usen	Zenith
Virulence phenotype Group 1	R	R	R	S	R
Virulence phenotype Group 2	R	R	R	R	R
Virulence phenotype Group 3	R	R	R	S	S
Virulence phenotype Group 4	S	S	S	S	S
Virulence phenotype Group 5	R	R	R	R	S
Virulence phenotype Group 6	R	R	S	S	R
Virulence phenotype Group 7	R	S	R	S	R

[1] Virulence reactions were characterized as R = resistant (mean disease rating $<$ 3.5); S = susceptible (mean disease rating $>$ 4.5).

Virulence diversity clearly indicates that virulence differences between the four predominant groups are much larger than within the groups (Table 2). A comparison of a number of isolates within each of the four MGR586 groups collected in a given season indicated that there was a clear correspondence between race and MGR586 group (Xia et al., 1993). However, when a broader group of germplasm is used as differentials, it is evident that considerable amount of variation in virulence

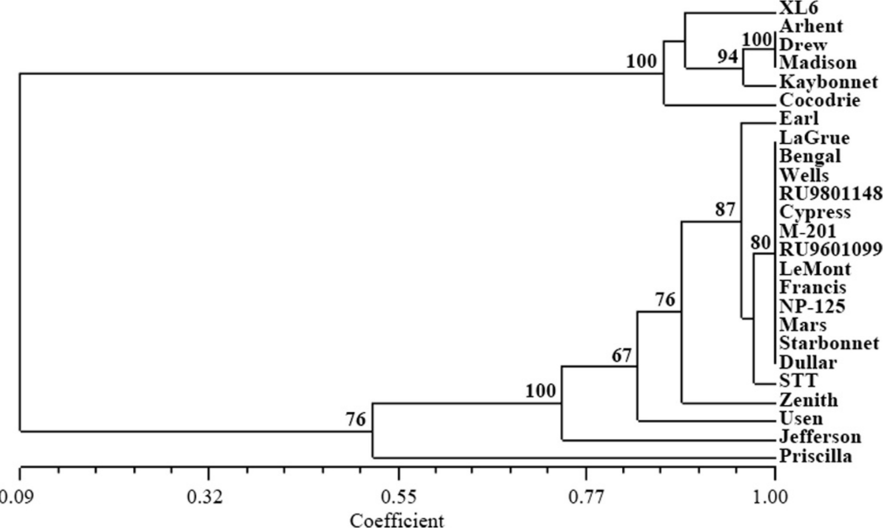

Fig. 2 Dendrogram showing disease reaction diversity among 25 rice genotypes. Dendrogram was based on combined data of 81 Group A isolates of P. oryzae and was generated with NTSYSpc version 2.11 using the UPGMA analysis. Bootstrap analysis was performed using Winboot software (www.irri.org/science/software/winboot.asp)

Table 4 Mean disease reactions of isolates from each of the MGR586 fingerprint groups on five commonly grown commercial cultivars (Wells, CL161, Coccodrie, Francis, and Bengal), a susceptible control (M201), and a Pi-ta containing cultivar (Banks) under greenhouse conditions

		Cultivar						
Isolate	MGR Group	M201 (Control)	Wells	CL161	Coccodrie	Francis	Bengal	Banks (*Pi-ta*)
DA3	A	6.2[1]	5.2	4.6	2.8	6.1	5.4	0.7
PH1	A	6.2	4.8	3.8	1.2	6.2	3.9	0.5
RT5	A	5.7	5.0	4.7	2.5	4.9	4.6	1.7
DA17	B	6.2	3.9	4.2	1.0	5.7	0.3	0.7
PH3	B	5.5	4.8	3.3	0.8	5.3	0.0	0.4
RT1	B	6.1	5.0	3.7	0.7	5.3	1.1	5.9
ZN17	C	4.8	3.9	3.4	1.0	4.0	2.3	0.0
BM1-24	C	4.1	3.9	4.1	2.9	4.2	3.0	0.2
A119	C	4.6	3.3	3.3	1.1	4.6	0.6	0.2
DR1	D	4.6	3.4	0.3	0.9	4.3	1.8	2.7
DR7	D	5.7	4.3	0.3	0.3	4.8	2.2	2.4
DR9	D	4.9	4.3	0.2	0.6	4.0	2.5	1.9

[1] Disease reactions are the mean disease ratings from three independent inoculation tests using 4 replications per test.

occurs among members of a given group, including Group A which has predominated based on sampling the population since 2000 (Tables 1 and 3). Also, some cultivars used as differentials are more similar to others in their overall susceptibility to various isolates and likely reflect similarities in both major and minor resistance gene combinations (Fig. 2).

It is somewhat difficult to hypothesize why Group A isolates have become so predominant in the population in Arkansas, particularly in more recent years. Based on virulence alone, representative isolates in all 4 groups are capable of infecting the five most commonly grown commercial cultivars under greenhouse conditions (Table 4). These five cultivars represent over 80% of the acreage grown in Arkansas since 2000 (Lee et al., 2006). However, it is obviously difficult to extrapolate susceptibility under greenhouse conditions with how given isolates may perform under field conditions. It is likely that Group A isolates have a number of fitness parameters in addition to virulence that makes them predominate in the population.

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Studies on the Complementary Differential Varieties and Local Physiologic Races of *Magnaporthe grisea* in Sichuan Province

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Abstract Chinese national differential varieties have failed to provide clues in virulence changes for the two re-surges of rice blast disease causing severe losses in Sichuan of China since 1986, where hybrid rice has been the major crop since 1980. So Lijiang Xintuan Heigu (LTH) and 5 restorer lines as resistance donors of hybrid rice, IR24, Minhui 63 (MH63), Duohui No 1 (DH1), Chenghui 448 (CH448), Neihui 99-14 (99-14) and RHR-1, were used as local differential varieties. In 2005–2006, 243 isolates of *Magnaporthe grisea* were differentiated into 41 virulent types, of which, 18 were of an annual frequency higher than 2.0% and then respectively named as local physiologic races CB0-CB17. A higher differentiation ability of Sichuan differentials than that of national ones but no distinct links between the races differentiated by two sets of differentials were observed. Based on the virulence spectrums of individual local races and the history of sequential release and breakdown of resistance in the restorer lines, the evolution of virulence of *M. grisea* in Sichuan was briefly described. Local races virulent to hybrid rice evolved from avirulent race CB0 and successively acquired their virulence to IR24, MH63, DH1, CH448 or (/and) 99-14. All of the races virulent to DH1, CH448, 99-14 and RHR-1 evolved from races virulent to MH63 but virulence to IR24, DH1, CH448 and 99-14 could be bypassed during the evolution. In 2006, the total frequency of CB5, CB14, CB16 and CB17, each virulent to DH1 and over 80 tested hybrid rice, reached 20.3%, which indicated a possible turn for the worse of the blast disease epidemic.

Keywords *Magnaporthe grisea* · Physiologic races · Differential varieties · Hybrid rice · Virulence

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1 Introduction

Rice blast caused by *Magnaporthe grisea* is the most important disease threatening the stability of the output of paddy fields over 1.5 million hm^2 in Sichuan Province of China, of which over 80.0% have been planted with hybrid rice since 1980. Sequential release of restorer lines conferring resistance to the blast disease in hybrid rice combinations had been experienced in Sichuan since 1978 (Peng et al., 1995). IR24, Minghui 63, Duohui No.1, Chenghui 149, Chenghui 177 and its sibling line Chenghui 448, Neihui 182, Shuihui 527, Neihui 99-14 were respectively released since 1979, 1986, 1995, 1996, 1998, 2000, 2001, and 2003 (Dr G. J. Ren at Sichuan Academy of Agricultural Sciences, personal communication). Due to the low number of the available male sterile and restorer lines at the beginning of hybrid rice popularization, the genetic background of the hybrid rice in Sichuan, as well as in other parts of China, was over-simplified. The annual acreages of Shan You No. 2 (Zhenshan 97A × IR24) in 1979–1985 and the hybrid combinations of Minghui 63 to Zhenshan 97A and other male sterile lines in 1986–1996 were respectively over $0.6–1.6 \times 10^6$ hm^2 in Sichuan. The breakdown of resistance in Shan You No. 2 since 1983 and Minghui 63 since 1989 has resulted in the unprecedented loss in rice yield of $4.3–4.5 \times 10^5$ tons (Peng et al. 1995, 1998, 1992) and the termination of their popularity in the fields. The popularization of breeding technologies of hybrid rice gave rise to over 200 hybrid rice combinations currently planted in Sichuan, of which, over 90.0% were susceptible to the disease according to our observation. The breakdown of resistance in Duohui No. 1 and Shuhui 527 since 2002 was followed by the third great epidemic in 2003–2005 and the termination of the popularization of the former in 2006.

The physiologic races of *M. grisea* differentiated by the China national differential varieties before the popularization of hybrid rice in Sichuan were dominated by Race ZG_1 (Peng et al. 1995; Ji et al. 2005), which was then replaced by races in ZB groups since 1983. The changes of in the most frequent races from ZB13, ZB15 to ZB29, ZB 31 and others in the group were not indicative of the subsequent resistance breakdown. Isolates virulent to Minghui 63 belonged to races in the different groups including ZA, ZB, ZC, ZD, ZE, ZF as well as ZG and were nominated as Minghui 63-virulence strains (Peng et al. 1995, 1998; Ji et al. 2005). Because of defects of the national differential hosts in the respect of the unclear genetic background, poor differentiating ability and the limited geographic application, etc. (Peng et al. 1995; Ling et al. 2000, 2004;), detection of virulence changes in different populations of *M. grisea* by laboratories all over China was conducted mainly by measurement of virulence frequencies to varieties planted in different areas (He et al. 1988; Li et al. 2005). Virulence frequencies could reflect the changes in the efficiency of resistant varieties, but they failed to indicate links among virulences to different varieties, and therefore could lead to neglecting of new trends in virulence changes. Practically, the arbitrary selection of the varieties out of the numerous varieties of unclear genetic background for the measurement of virulent frequency is prone to result in inconsistency of the data over the years and areas. In the case of the diversification of planted varieties, nomination of the strains virulent to different varieties of

unknown resistance genetics becomes complicated, if not impossible. To enhance the differentiating ability of the national differential hosts, local complementary differentiating host varieties from regional varieties were set up in Jiling, (Cao et al. 1983; Liu et al. 2002) Fujian, (Zhang & Yu 1981; Gan et al. 1986). Ningxia, (Mu & Gu 1985) and other provinces. The Japanese differential varieties with single genes and their isogenic line ascendants were used for virulence detection in the areas where the planted varieties were mainly of the japonica rice type (Luo et al. 2000; Lei et al. 2002; Xiong et al. 2005).The problems in predicting resistance breakdown in hybrid rice using the national differentials and identification of a "super strain" of *M. grisea* virulent to numerous restorer lines (Ji et al. 2005) suggested the urgent need for new varieties with improved differentiating abilities. Lijiang Xintuan Heigu and the restorer lines of past or currently resistant hybrid rice combinations planted in large areas in Sichuan (such as IR24, Minghui 63, Duohui No. 1, Neihui 99-14, Chengui 448 as well as RHR-1, a newly bred resistant restorer line) were selected as local complementary differentiating hosts, and the consequently differentiated Sichuan races were reported in this paper. Differences in the differentiating abilities between national and local varieties and an indication of the virulence changes to the prevalent hybrid rice combinations were found.

2 Materials and Methods

2.1 Rice Varieties

The China national differentiating varieties, Tetep, Zhenglong 13, Sifeng 43, Hejiang 18, Dongnong 363, Guandong 51 and Lijiang Xintuan Heigu (LTH), were provided by the Institute of Plant Protection under Zhejiang Academy of Agricultural Sciences (Hangzhou, China); Duohui No. 1 (DH1) and Neihui 99-14 (99-14) were provided by Neijiang Institute of Agricultural Sciences (Neijiang, China), IR24, Minghui 63 (MH63), Chenghui 448 (CH448) were obtained from Crop Science Institute under Sichuan Academy of Agricultural Sciences (Chengdu, China), RHR-1 was provided by Prof. H. C. Kuang at Rice and Sorghum Research Institute under Sichuan Academy of Agricultural Sciences (Luzhou, China). Commercial seeds of hybrid combinations were provided by the authenticated seed companies.

2.2 Isolates of M. grisea and Inoculation Assays

Respectively, 137 and 84 samples of diseased panicles were collected throughout the province in 2005 and 2006. After immersion in sterile water for 3 hrs, diseased segments without grain were incubated in darkness at room temperature for 12–24 h. In 2005 and 2006, respectively 115 and 128 isolates derived from single conidiospore were obtained in accordance to the methods of Cai and Jin (Chai & Jin 1991) and

were kept in tubes of PDA agar at 27 °C for temporary use and on filter papers at −20 °C for permanent storage.

For sporulation, five mycelium discs, 5 mm in diameter, for each isolate were transferred onto plates of PDA-rice meal medium (200 g of potato, 40 g of rice meal, 18 g of agar in 1000 ml H_2O, pH 6.8–7.0) and incubated at 27 °C for 5 days in darkness. Mycelia were rubbed off using sterile slides and plates were illuminated by fluorescent light for 2 days at 27 °C. Conidiospores were washed off and filtered through two layers of cheesecloth. The concentration of spores was adjusted to 5–10 × 10^4 spores/ml before 1/10^4 (v/v) of Tween 20 were added for inoculation.

Seedling management and inoculation were conducted according to methods of All China Coorporation of Research on Physiological Races of *Pyricularia Oyzae* (1980).

3 Results

3.1 Virulent Types and Physiologic Races Identified by Sichuan Differential Varieties

During 2005 and 2006, 243 isolates were differentiated into a total of 41 virulent types by LTH, IR24, MH63, DH1, CH448, 99-14 and RHR-1 as local complementary differential varieties. In 2005, 26 virulent types were obtained from 115 isolates and, in 2006, 38 virulent types were obtained from 128 isolates. There were 18 virulent types of which the annual isolation frequencies were higher than 2.0%, and these were consequently nominated as local physiologic Races from CB0 to CB17 (Table 1). The frequency of isolation of Sichuan Races CB2 (19.1%) and CB3 (17.3%) were highest in 2005 and 2006, respectively. The other major Sichuan races in 2005 were CB5, CB3 and CB4 respectively with a frequencies at 14.4%, 12.2% and 12.2%. The annual frequencies of other Sichuan races were lower than 10.0% (Fig. 1). Sichuan Race CB1 wasn't detected in 2006 while CB16 and CB17 only occurred in 2006. The occurring frequencies of CB6, CB9, CB11, CB13, B14, and CB15 increased higher than 2.0% in 2006 and they consequently obtained a race nomenclature.

3.2 Comparison of Differentiating Ability Between China National and Sichuan Local Differential Varieties

The preceding 128 *M. grisea* isolates in 2006 were differentiated into 19 physiologic races in groups ZA, ZB, ZC, ZE using the China national differentials (Fig. 2), which was much lower than the number of virulent types differentiated by Sichuan differentials. Comparison of the races identified by the national and Sichuan differentials indicated no distinct links between these two sets of races (Table 2).

Table 1 Virulent types and local physiologic races of *M. grisea* in 20005–2006 from Sichuan Province

Virulent *type*	Response to complementary differential varieties							Isolate no.		Local race
	LTH[1]	IR24	MH63	DH1	CH448	99-14	RHR-1	2005	2006	
1	S[2]	R	R	R	R	R	R	10	8	CB0
2	S	S	R	R	R	R	R	4	0	CB1
3	S	R	S	R	R	R	R	22	12	CB2
4	S	S	S	R	R	R	R	14	22	CB3
5	S	R	R	S	R	R	R	1	1	
6	S	R	S	S	R	R	R	14	2	CB4
7	S	S	S	S	R	R	R	17	9	CB5
8	S	R	S	R	S	R	R	2	2	
9	S	S	R	R	S	R	R	0	1	
10	S	S	S	R	S	R	R	2	3	CB6
11	S	R	R	S	S	R	R	0	1	
12	S	R	S	S	S	R	R	1	1	
13	S	S	S	S	S	R	R	1	1	
14	S	R	R	R	R	S	R	0	1	
15	S	R	S	R	R	S	R	3	4	CB7
16	S	S	S	R	R	S	R	3	4	CB8
17	S	R	S	S	R	S	R	1	2	
18	S	S	S	S	R	S	R	2	3	CB9
19	S	R	S	R	S	S	R	3	1	CB10
20	S	S	S	R	S	S	R	1	3	CB11
21	S	R	S	S	S	S	R	0	1	
22	S	S	S	S	S	S	R	1	1	
23	S	R	R	R	R	R	S	0	1	
24	S	S	R	R	R	R	S	0	1	
25	S	R	S	R	R	R	S	3	2	CB12
26	S	S	S	R	R	R	S	2	7	CB13
27	S	R	R	S	R	R	S	1	0	
28	S	R	S	S	R	R	S	2	0	
29	S	S	S	S	R	R	S	1	6	CB14
30	S	R	R	R	S	R	S	0	1	
31	S	R	S	R	S	R	S	1	1	
32	S	S	S	R	S	R	S	1	6	CB15
33	S	R	S	S	S	R	S	0	1	
34	S	S	S	S	S	R	S	0	1	
35	S	R	S	R	R	S	S	0	1	
36	S	S	S	R	R	S	S	0	2	
37	S	R	S	S	R	S	S	0	1	
38	S	R	S	R	S	S	S	2	2	
39	S	S	S	R	S	S	S	0	7	CB16
40	S	S	S	S	R	S	S	0	1	
41	S	S	S	S	S	S	S	0	4	CB17

[1] LTH: Lijiang Xintuan Heigu, MH63: Minghui 63, DH1: Duohui No. 1, CH448: Chenghui 448, 99-14: Neihui 99-14.
[2] S: susceptible, reaction types: 3-4, R: resistant, reaction types: 0-2.

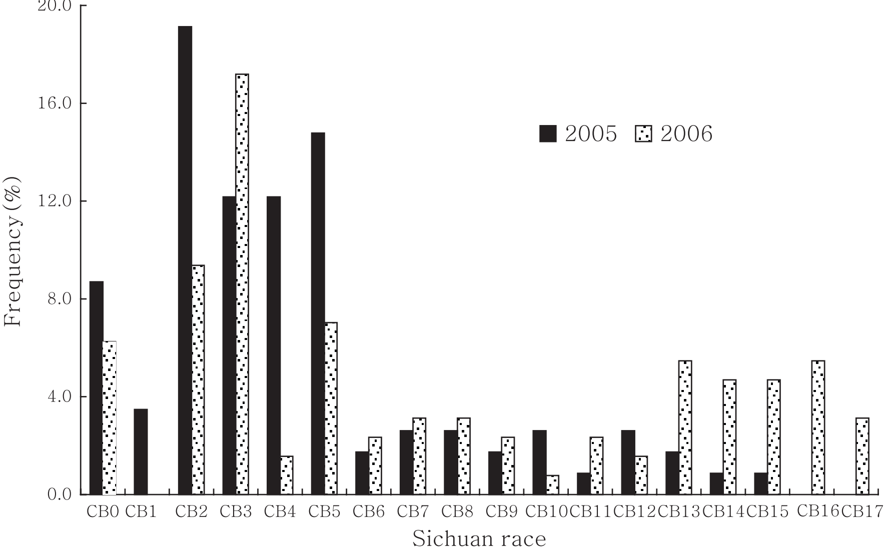

Fig. 1 Occuring frequncies of Sichuan physiological races of *Magnaporese grisea* in 2005 and 2006 identified by complementary differential varieties

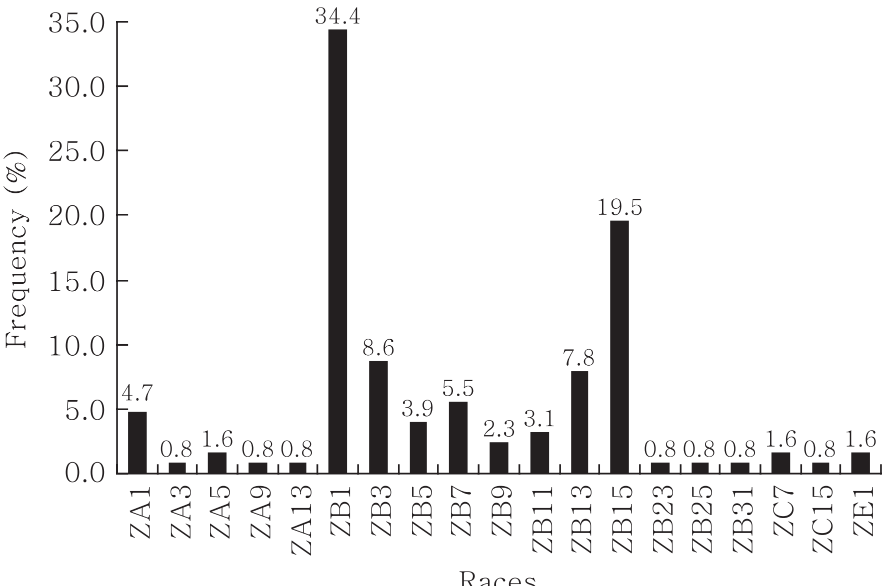

Fig. 2 Occurring frequencies of physiological races of *M. grisea* in 2006 from Sichuan Province identified by Chinese national differential varieties

Table 2 Comparison of physiological races of *M. grisea* in 2006 from Sichuan Province identified by Chinese national and complementary Sichuan differential host varieties

Sichuan races	Races identified by national differential varieties
CB0	ZE1, ZB25, ZB15, ZB7, ZB1, ZA9, ZC15
CB2	ZB23, ZB15, ZB7, ZB13, ZB5, ZA1
CB3	ZB7, ZB15, ZB11, ZB7, ZB3, ZB13, ZB5, ZB1, ZA5
CB4	ZB13, ZB1
CB5	ZB15, ZB11, ZB1, ZA13
CB6	ZB13, ZB9, ZB1
CB7	ZB31, ZB13, ZB1
CB8	ZB15, ZB5
CB9	ZB15, ZB1, ZA1
CB10	ZB1
CB11	ZB15, ZB5, ZB1
CB12	ZB15, ZB3
CB13	ZB9, ZB1, ZA1
CB14	ZB7, ZB3, ZB1, ZA1,
CB15	ZB15,ZB1
CB16	ZB1
CB17	ZB1

3.3 The Evolution of Virulence to Hybrid Rice in Sichuan

The evolution of virulence to hybrid rice since its popularization in Sichuan was postulated on the basis of the virulence spectrums of individual Sichuan races and the sequence of field release of different restorer lines among the complementary differential varieties. From the Sichuan Races CB0, mainly virulent to conventional rice varieties released in 1970s, the majority of Sichuan races subsequently obtained virulence to IR24 released in 1979 and eradicated since 1986, MH63 released in 1986, DH1 released in 1995, CH448 (and/or) 99–14 which were respectively released in 1998 and 2003 and are still resistant in the fields. A small fraction of Sichuan races were virulent to RHR-1 that was released in 2005 as a resistance donor for breeding. During the evolution, the virulence to the differentials released later than MH63 was always accompanied by that to MH63 whereas virulence IR24, DH-1, Ch448 and 99-14 could be bypassed by some races (Fig. 3).

3.4 Virulence of Races to Major Hybrid Rice Combinations in Sichuan Province

Virulence frequencies of different Sichuan races to 101 hybrid rice combinations were obtained after the inoculation in 2006. The virulence frequency of Sichuan Race CB0 to 21 combinations were higher than 50.0% while that to other 80 tested combinations was not higher than 33.3%. No virulent isolates in CB0 was detected to 24 combinations. The number of the hybrid combinations to which a virulent frequency higher than 50.0% was found in other tested Sichuan Races varied from

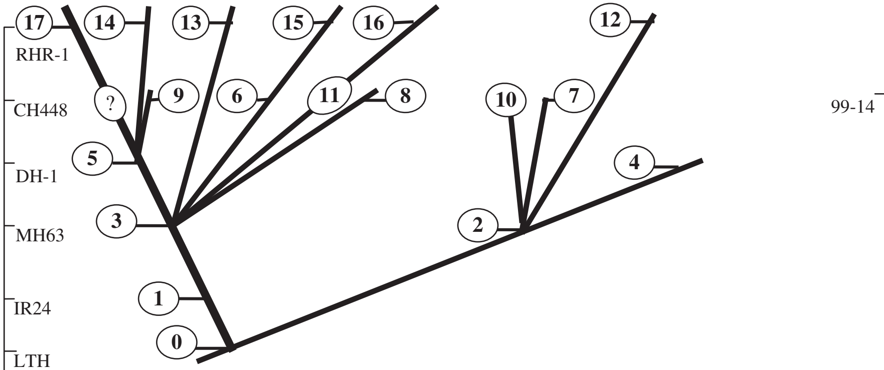

Fig. 3 The evolution tree of the virulence of different Sichuan races of *Magnaporthe grisea*. Abbreviations for differential varieties of rice: Lijiang Xintuan Heigu (LTH), Duohui No. 1 (DH-1), Neihui 99-14 (99-14) Minghui 63 (MH63), Chenghui 448 (CH448)

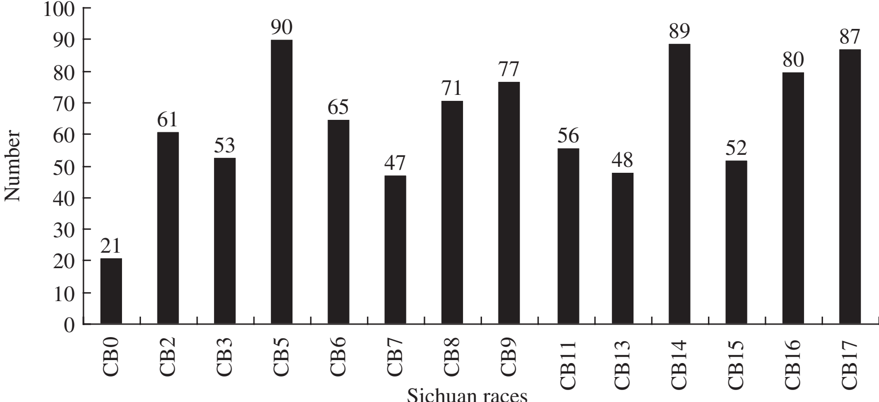

Fig. 4 The number of hybrid combinations to which the virulence frequencies of the tested Sichuan races of *Magnaporthe grisea* were at or higher than 50.0%

47 to 90 (Fig. 4). Such number of Races CB5, CB9, CB14 and CB17, all virulent to DH1, was 90, 77, 89 and 87, respectively. The virulence of each tested Sichuan race to II You 734, Yixiang 1313, Yixiang 725, II You 1577, Gang You 26, Gang You 3551, II You 802, Gang You 615, Zhong You 936, II You 949, Chuanfeng No. 7 and Gang You 19 were all higher than 50.0% whereas that of Lu You 502 were all lower than 33.0% in 2006.

4 Discussion

After extensive planting of hybrid rice in Sichuan since 1980, the majority of isolates of *M. grisea* including parts of isolates sampled from conventional rice were virulent to hybrid rice. While the virulence to the less popular restorer lines such as IR24,

DH-1, CH448, 99-14 may be bypassed during evolution, the accompanied virulence to MH 63 of the races virulent the to subsequently released restorer lines was in accordance to the fact that combinations derived from MH63 were continuously planted up to now in Sichuan since 1986 and their annual acreages were larger than 65.0% of total rice areas during the first 10 years (Peng et al. 1995, 1998, 1992).The total frequency as high as 20.3% of Sichuan Race CB5, CB14, CB16, and CB17, which were virulent DH1 and the majority of the currently prevailing hybrid combinations explained their susceptibility in the fields and indicated probable enhanced levels of disease epidemics in Sichuan if the weather conditions allow. CB17, virulent to all the differential hosts including the released resistance source RHR-1, should have special attention in screening for resistance lines in breeding.

The development of international differential varieties of mono-isogenic lines is one of the common efforts of different research teams of *M. grisea* physiologic races in the world (Ling et al. 2004), while such efforts might not exclude the necessity to develop local complementary differential hosts for fast and direct detection of virulence changes to assorted local varieties of unknown resistance genetics. Such necessity was supported by the stronger differential abilities of Sichuan complementary differential varieties than the China national differential varieties and evident indication of the evolution of virulence of *M. grisea* after the popularization of hybrid rice in Sichuan. The further dissection of the resistance genetics of the local complementary differential varieties and their transformation into mono-isogenic lines by LTH as the backcrossing parent can be an important part of the common efforts toward international differential varieties. Standard sampling from the sample nurseries of the same varieties, strict control of the seed purity and seedling physiologic conditions as well as the post-inoculation temperature and humidity would also improve the physiologic race research. In this paper, the seed purity of hybrid rice and environment conditions after the inoculation might have affected the precision of the virulence frequency measurement. *In vitro* inoculation of seedlings cultured by nutrition solutions in a controlled environment is currently underway for further improvement.

The nomenclature for Sichuan races of *M. grisea* was numeric and an occurrence frequency over 2.0% was required for the nomination of a virulent type as Sichuan race. Further requirement of its distinct virulence to a certain number of varieties planted in Sichuan and an agreement among the researchers on the nomination of physiologic races in the province will help to extend the information to plant breeders and plant protection agencies and to avoid the possible arbitrary mistakes.

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Blast Race Monitoring for Stable Use of Blast Resistance in Rice

Shinzo Koizumi

Abstract Differential systems of blast races based on the gene-for-gene relationship between host and pathogen were established in Japan by Yamada et al. (1976) and Kiyosawa (1984), and blast race monitoring was conducted nationwide in Japan in 1976, 1980, 1994, and 2001 using these systems. Those monitoring results showed that the frequencies of complete resistance genes in cultivated rice cultivars affected the prevalence of the corresponding avirulence genes in blast races, and such monitoring results have contributed to decisions regarding component proportions in three released Japanese multilines, as well as the development of rice cultivars with high levels of partial resistance. However, the optimal way for stable use of blast resistance in rice is not yet fully understood, because sufficiently quantitative analyses of blast race increases in host populations with different resistance genes, which are required to understand the optimal way, have not been conducted. For conducting the analyses, development of simple methods is necessary for monitoring blast races relative to resistance genotypes of the rice cultivars. These methods will facilitate accumulation of quantitative data on the increases of blast races in host populations with different resistance genes and such data will contribute to constructing reliable epidemiological simulation models to simulate blast race increases in the host populations.

Keywords Monitoring · Blast races · Rice · Resistance · Stable use

1 Introduction

Blast race monitoring has been conducted using different differentials in various countries (Kobayashi et al. 2007). However, the stability of blast resistance in rice cultivars has only been empirically determined in those countries because the

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complete and partial resistance genotypes of the cultivated rice cultivars have not been known. In contrast, in Japan, after breakdowns of complete resistance to blast in rice cultivars during the 1960s, complete resistance genotypes and partial resistance levels for most of the cultivated rice cultivars have been clarified and blast race monitoring has been conducted. However, realizing stable use of blast resistance has not been achieved because sufficiently quantitative analyses of blast race increases in host populations with different resistance genes have not been conducted. This paper describes blast race monitoring for realizing stable use of blast resistance in rice.

2 The Development of Differential Systems in Japan

After the initial discovery of blast races by Sasaki (1922) and breakdowns of complete resistance in many rice cultivars in the 1960s, several differential systems for blast races were developed and blast race distribution was monitored in Japan. The first differential system was developed by Goto et al. (1964), and an international differential system was developed by US-Japan cooperative research in 1967 (Goto et al. 1967).

These differential systems, however, had been developed without consideration of the gene-for-gene relationship between host and pathogen. Alternatively, Kiyosawa (1974) had conducted gene analyses of blast resistance and identified many resistance genes in rice cultivars. Based on those gene analyses and the gene-for-gene relationship between resistance in rice and avirulence in blast fungus, Yamada et al. (1976) and Kiyosawa (1984) developed new differential systems, which are currently used for blast race monitoring in Japan.

3 Monitoring and Analyses of Race Distribution in Japan

Using the above-mentioned systems, blast race distribution was monitored nationwide in Japan in 1976, 1980, 1994, and 2001 (Yamada et al. 1979; Yamada 1985; Naito et al. 1999; Koizumi et al. 2007). The procedure for the blast race monitoring basically followed that of Yamada et al. (1979), which was decided by analyses of the distribution data of both blast races and cultivated rice cultivars in central Japan's Niigata prefecture.

The procedure is as follows: First, one paddy field is selected from approximately 1,000 ha or 4,000 ha paddy fields with randomized systematic sampling. Second, one blast lesion per 1,000 ha fields is, or four lesions per 4,000 ha fields are, collected from that field. Third, after mono-conidial isolation from the lesions, blast races of the isolates are identified with spray-inoculation using Japanese differentials.

The number of blast races differentiated in such monitoring from 1976 to 2001 with the nine Japanese differentials was 15–23 among 1,050–2,376 isolates in

Table 1 Numbers and diversities of blast races, and sampling densities for blast race monitoring in Japan in 1976, 1980, 1994, and 2001

	1976	1980	1994	2001
Number of isolates	2,245	2,376	1,526	1,050
Number of races	23	22	23	15
Shannon index	1.35	1.51	1.53	1.41
Rice cultivated ha per one isolate	1,221	989	1,442	1,590
Rice cultivated ha	2,741,000	2,350,000	2,200,051	1,699,645

respective years (Table 1). In 1979 and 1980, the predominant race was 003 (virulent to the resistance gene *Pia*) and races 007, 033, 001, and 103 followed that. In contrast, in 1999 and 2001, race 007 (virulent to the gene *Pii* and *Pia*) predominated, followed by races 001, 003, 005, 037, and 033 (Fig. 1).

The monitoring results showed that the prevalence of blast races was affected by the frequencies of corresponding complete resistance genes in cultivated rice cultivars (Fig. 1) and indicated that statistically significant positive correlations existed between isolation frequencies of blast races virulent to *Pia, Pii, Pik, Pita*, and *Piz* and the frequencies of the genes in cultivated rice cultivars (Fig. 2).

Cluster analysis was conducted using the data set of isolation frequencies of blast races virulent to *Pia*, *Pii*, *Piz*, and *Pita* in 1976, 1980, 1994, and 2001. This analysis divided 37 prefectures with full data sets from those years, among the 47 prefectures that comprise Japan, into five groups and three prefectures. Each of these groups or prefectures represented one distinctive feature of the changes in the blast races, and variations in the isolation frequencies of the blast races virulent to the genes in each of the groups or prefectures corresponded to those in cultivated percentages of rice cultivars possessing the genes.

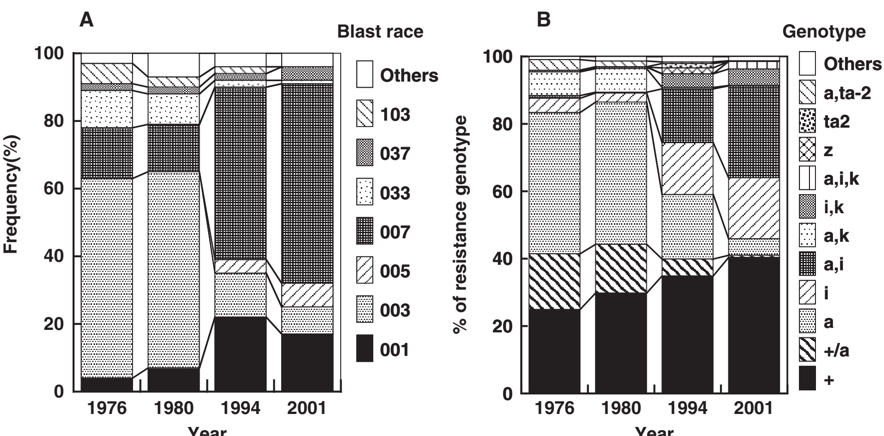

Fig. 1 Changes in frequencies of main blast races (**A**) and percentages of blast resistance genotypes in cultivated rice cultivars (**B**) in Japan from 1976 to 2001

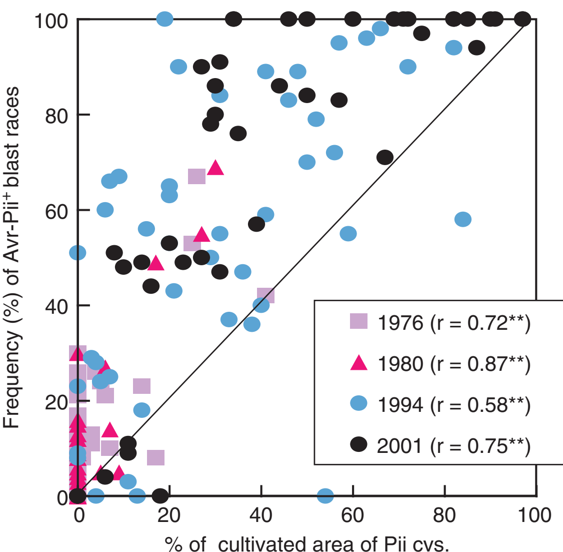

Fig. 2 Relationship between percentages of cultivated areas of rice cultivars containing *Pii* and isolated frequencies of $Avr\text{-}Pii^{+}$ blast races in Japan. Each symbol represents values of the percentage and frequency in a prefecture in a monitoring year

4 Factors Affecting the Prevalence of Blast Races

Besides the complete resistance genotypes of cultivated rice cultivars mentioned above, several factors affecting the prevalence of blast races can be considered. They are levels of partial resistance in the host, changes in rice cultivars with different blast resistance in scale and time, fitness cost of virulence, variations in virulence, genetic drift and gene flow in the pathogen, environmental conditions, cropping system, crop management and other variables (Mundt 2002).

High levels of partial resistance to blast in cultivated rice cultivars reduced the prevalence of blast races virulent to *Pii* in small districts in central Japan's Aichi prefecture (Koizumi, unpublished). The aggressiveness (fitness) of blast races is also assumed to affect their prevalence. The author examined the aggressiveness of two blast races, 003 and 033, whose isolates were collected by Yamada et al. (1979) when they monitored blast race distribution nationwide in Japan in 1976. Race 003 is unable to attack *Pik*, whereas race 033 can. The aggressiveness of race 003 on Norin 29, which has no complete resistance gene against most Japanese isolates, was stronger than that of race 033 (Table 2; Koizumi 1982). In central Japan's

Table 2 Difference in aggressiveness of blast races 003 and 033 on Norin 29

Race	Lesion area (mm^2)	Sporulation ability (No. of formed spores/lesion)
003 ($Avr\text{-}Pia^{+}$)	25.8**	9.8×10^{3}*
033($Avr\text{-}Pia^{+}$, $Avr\text{-}Pik^{+}$)	21.9	8.3×10^{3}

* and ** denote statistically significant at $P < 0.01$ and $P < 0.05$.
Values indicate means of 22 isolates belonging to the respective races.

Niigata prefecture, isolation frequencies of race 033 decreased after a reduction in cultivated areas of rice cultivar including *Pik* during the 1970s (Yaoita et al. 1977). The lower aggressiveness of race 033 probably affected the decrease in the race isolation frequency.

Virulence mutation rates of the rice blast fungus are estimated to be relatively high, calculated at ten to the power minus three to five (Kiyosawa 1976). Point mutation, deletion and transposon insertion, and parasexual recombination are all thought to cause virulence variation (Koizumi 2007; Noguchi et al. 2006). Virulent fungal mutants are commonly considered to have lower fitness compared with the original isolates. However, Fujita et al. (2007) reported that fitness levels of the variants differed between two variants and that the existence of stabilizing selection was not yet proven. Further studies are needed to determine whether universal features exist among blast races for fitness and other variables affecting race prevalence.

5 Realzing Stable Use of Blast Resistance Through Blast Race Monitoring

Several methods have been proposed for realizing stable use of blast resistance (Kiyosawa 1985). These include mixtures of cultivars or near-isogenic lines with differing complete resistance; complete resistance gene rotation; complete resistance gene pyramiding; use of partial resistance; and the accumulation of complete and partial resistances. Kiyosawa (1985) conducted a theoretical comparison among the methods for clarifying resistance stability. However, the optimal method for stable use of blast resistance is not yet clear because sufficiently quantitative analyses of blast race dynamics in host populations with different resistance genes have not been performed. For the quantitative analyses of race changes, it is necessary to accumulate epidemiological data on blast race prevalence. Simple methods of race identification, including seedling traps, should be developed to accumulate such data.

Conventional blast race identification is laborious, including monoconidial isolation of the fungus, inoculation of differentials, and conducting phenotype evaluation. Many avirulence genes, including cloned *AVR-Pita*, *AVR-Co39*, and *ACE1*, have been identified in the fungus, and DNA markers linked to those avirulence genes have been confirmed (Koizumi 2007). Development of DNA makers tightly linked to the avirulence genes are desirable for easy monitoring of blast races.

6 Development of Epidemiological Models

Ashizawa (2005) developed BLASTMUL, an epidemiological computer simulation model, from BLASTL, a systems analytical model for leaf blast epidemics, to simulate short term leaf blast development in multilines. BLASTMUL simulation can mimic leaf blast epidemics in ‘Sasanishiki’ and ‘Koshihikari’ multilines.

The model includes parameters for virulence mutation rates, fitness cost, levels of partial resistance, and other variables, and can quantitatively simulate epidemics of respective blast races after setting initial and incorporated amounts of inocula for respective blast races. The model is intended to simulate blast epidemics in rice populations with different blast resistance genes, as well as multilines. The model will likely contribute to clarifying the realization of stable use of blast resistance after the model has added more reliable parameters, as estimated by the accumulated epidemiological data.

Kiyosawa (1985) conducted theoretical comparisons among the different methods for the utilization of blast resistance to clarify their stability using mathematical models, and Sasaki (2000) mathematically analyzed host-parasite coevolution in a multilocus gene-for-gene system. It is conceivable that such analyses with mathematical models will be able to contribute to stable use of blast resistance. However, reliable parameters are still lacking. Improving models with the reliable parameters estimated from accumulated data will also contribute to declaring stable use of blast resistance.

7 A Global Differential System

The IRRI-Japan Collaborative Research Project recently developed new differentials that are composed of LTH monogenic lines with 24 single complete resistance genes (Kobayashi et al. 2007); Hayashi and Fukuta (2007) proposed a new international system for differentiating blast races using these new differentials. Some Japanese differentials have additional genes, such as *Pish*, in addition to the target genes, and these additional genes can mask reactions of the target genes to blast isolates, especially from the tropical region. The new differentials developed by the IRRI-Japan Project do not include such genes. The system will contribute to effective use of blast resistance globally through accumulation of race monitoring data.

8 Complete Resistance Genotypes and Partial Resistance in Rice Cultivars

Complete resistance genotypes and partial resistance levels for most rice cultivars in Japan are already known. However, information about the blast resistance of rice cultivars in many other countries is limited, even though such information is necessary for epidemiological analyses of the prevalence of blast races. Standard blast isolates to determine complete resistance genotypes of rice cultivars, and development of simple molecular methods for detecting resistance genes, such as tightly linked DNA makers, are urgently needed for analyses in these countries.

Partial resistance also affects the prevalence of blast races, and the monitoring of partial resistance levels is important for stable use of blast resistance. Recent quantitative trait loci (QTL) analyses identified many partial resistance blast genes,

including cloned *pi21*, together with their putative chromosomal locations and linked DNA markers (Koizumi 2007). DNA markers tightly linked to partial resistance genes can detect those genes relatively easily and estimate levels of partial resistance in cultivars whose levels are currently unknown. This information can improve the accuracy of blast race prevalence analyses.

Partial resistance to blast is generally non-race specific. However, Zenbayashi-Sawata et al. (2005) recently found a new gene-for-gene relationship between isolate-specific partial resistance gene *Pi34* and an aggressiveness gene in the blast pathogen by the crosses between the host and pathogen. Partial resistance with similar characteristics to *Pi34* must be checked for stable use of blast resistance.

9 Conclusion

New differentials developed by the IRRI-Japan Collaborative Research Project have made both global surveys of the distribution of blast races and estimation of complete resistance genotypes of rice cultivars possible. However, stable use of blast resistance is not yet accomplished because sufficiently quantitative analyses of increases in blast races, which are required to attain such stability, have not been sufficiently performed. Development of simple monitoring methods for blast races as well as resistance genotypes of rice cultivars using DNA markers tightly linked to avirulence or resistance genes can accelerate the accumulation of epidemiological data on population interactions between the host and the pathogen. Analyses of such accumulated data will contribute to construction of reliable epidemiological simulation models that can guide stable use of blast resistance through simulations of blast race increases in host populations with different resistance genes.

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Pathogenicity-Related Compounds Produced by Blast Fungus

Tetsu Tsurushima, Hitoshi Nakayashiki, Yukio Tosa and Shigeyuki Mayama

Abstract For over a hundred years, it has been discussed whether or not *Pyricularia* isolates on rice (*Oryza sativa*) and on crabgrass (*Digitaria sanguinalis*) belong to the same species, *Pyricularia grisea* (teleomorph, *Magnaporthe grisea*). We thought that pyrichalasin H, isolated from a *Digitaria* isolate, could be used as a marker of the chemotaxonomy of *Pyricularia* isolates. Pyrichalasin H was only detected in culture filtrates of *Pyricularia* isolates to infect *Digitari*a plants among 72 isolates from 20 species of gramineous plants. There was a correlation between pyrichalasin H production and the ability of *Pyricularia* isolates to infect *Digitaria*. Pre-treatment of leaf sheaths of crabgrass with pyrichalasin H led to penetration and colonization by nonhost isolates. Thus, we propose that pyrichalasin H may be responsible for the specific pathogenicity of *Pyriculari*a isolates on the *Digitari*a genus. This result also supports the concept that isolates from rice and other cereals should be referred to as *Magnaporthe oryzae* and isolates from *Digitaria* should be referred to as *M. grisea*. The blast fungus produces necrotic lesions on its original host plant. These lesions might be formed by phytotoxins of blast fungus. Some necrosis-inducing factors have been isolated from *Pyricularia* isolates. However, their toxins have not been compared to their quantities in cultures of *Pyricularia* isolates and their activity on host plants. We searched for the necrosis-inducing factor from a *Triticum* isolate pathogenic on wheat (*Triticum aestivum*). Pyriculol and epipyriculol were detected as the main necrosis-inducing factors.

Keywords Crabgrass · Necrosis · Pathogenicity-related compound · Pyrichalasin H · Pyriculol

1 Introduction

Pyricularia isolates consist of several host-specific subgroups. Each of these subgroups produces typical blast lesions on the original host plant, but not on other plants (Kato et al. 2000). For example *Oryza* isolates from rice (*Oryza sativa*)

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produced blast lesions on rice, but not on foxtail millet, common millet and crabgrass (Table 1). Likewise, *Digitaria* isolates from crabgrass (*Digitaria sanguinalis*) and pangola grass (*Digitaria smutsii*) produced typical blast lesions on crabgrass but not on rice, foxtail millet and common millet. On the other hand, many isolates from other gramineous plants were able to infect Italian ryegrass.

Specificity of blast fungi to certain gramineous plants may result from the presence of not only avirulence factors, but also active factors such as host specific toxins (HSTs), which some fungal pathogens (particularly those in the genera *Alternaria* and *Cochliobolus*) produce as agents of pathogenicity (Kohmoto and Otani 1991; Walton 1996). Some phytotoxic metabolites have been isolated from cultures of *Pyricularia* isolates (Fig. 1). Pyriculol-related compounds [pyriculol, epipyriculol, pyriculariol, pyricuol, 3-(1′, 3′- pentadienyl)-3,4-dihydro-1*H*-2- benzopyran-4,8-diol and 4-(1′-hydroxy-2′-butenyl)-1,4-dihydro-2,3- benzodioxocin-10-ol] (Iwasaki et al. 1969; Nukina et al. 1981; Kim et al. 1998; Kono et al. 1991) and tenuazonic acid (Iwasaki et al. 1972) from *Oryza* isolates induced necrosis on the leaves of rice. Pyrichalasin H, isolated from a *Digitaria* isolate, inhibited the growth of rice seeds but not induce any visible symptoms in rice leaves (Nukina 1987). However, these toxins have not been compared for their effects on specific host plants. We have searched for the pathogenicity-related compounds of the blast fungus. In this paper, we first describe the production of pyrichalasin H and pathogenicity on *Digitaria* of 72 isolates among 20 gramineous plants. Then we discuss the role that this toxin might play in the specificity of blast fungus. Next, we report the main necrosis-inducers from *Pyricularia* isolates pathogenic on cereals.

2 Materials and Methods

2.1 Fungal Materials

A total of 72 isolates of *P. grisea* (Cooke) Sacc. (or *P. oryzae* Cav.) [teleomorph, *Magnaporthe grisea* (Hebert) Barr or *M. oryzae* Couch] from blast lesions of 20 gramineous species were used in this experiment. For long-term storage the isolates were grown on sterilized barley seeds in a vial, dried thoroughly at 25 °C and maintained at 5 °C in containers with silica gel, as a stock culture. For short-term storage, the isolates were grown on potato dextrose agar (PDA). All isolates were deposited at the laboratory of plant pathology, faculty of agriculture, Kobe University.

2.2 Detection of Pyrichalasin H in Culture

For each isolate of *P. grisea* (or *P. oryzae*), small plugs of mycelium grown from oatmeal agar cultures were inoculated into 50 mL of a soy sauce-sucrose medium (Nukina and Namai 1991) in a 200-mL Erlenmeyer flask and then grown at 25 °C for 10 days on a rotary shaker (110 rev/min). Mycelial mats were obtained by

Table 1 Pathogenicity of *Pyricularia* isolates to five gramineous plants[a]

		Infected type[b]				
Original host	Isolate	Rice[c]	Foxtail millet	Common millet	Crabgrass	Italian ryegrass
Rice (*Oryza sativa*)	0903-4	4*	0	0	0	4
	88A	4*	0	0	0	4
	IN77-32-1-1	4*	0	0	0	4
	IN77-32-1-3	4*	0	0	0	4
	IN77-46-1-3	4*	0	0	0	3
	NAGA68-138	4*	0	0	0	4
	UN05-1	4*	0	0	0	4
	UN05-2	4*	0	0	0	4
	ISHI5-1	4*	1	0	0	4
	CHNOS59-6-1	4*	1	0	0	3
	CHNOS59-8-1	4*	0	0	0	2
	Br10	4*	1	0	0	4
	Br11	4*	0	0	0	3
	Br13	4*	0	0	0	3
	Br14	4*	2	0	0	4
	Br15	4*	0	0	0	4
	Br18	4*	0	0	0	4
	PO-12-7302	4*	0	0	0	4
	CD276	4*	0	0	0	4
Foxtail millet (*Setaria italica*)	CFSI1-7-2	0	4	0	0	0
	NNSI3-2-1	0	4	0	0	0
	NRSI2-2-2	0	4	0	0	3
	NRSI3-1-1	0	4	0	0	2
	NRSI3-1-3	0	4	0	0	1
	IN77-16-1-1	0	4	0	0	4
	IN77-20-1-1	0	4	0	0	3
	IN-77-22-1-1	0	4	0	0	4
Common millet *(Panicum miliaceum*)	NNPM1-2-1	0	0	4	0	4
	STPM1-3-2	0	0	4	0	3
Crabgrass (*Digitaria sanguinalis*)	NI907	0	0	0	4	3
Pangola grass (*Digitaria smutsii*)	NI980	0	0	0	4	4

[a] Data was modified from a paper of Kato et al. (2000).

[b] 0, no infection; 1, minute, pinhead-sized spots; 2, small brown to dark brown lesions with no distinguishable centers; 3, small eyespot-shaped lesions with gray centers; 4, typical blast lesions elliptical with gray centers, usually coalescing; "4*", indicates susceptible or resistant, depending on cultivars.

[c] Plant tested: rice (nine Japanese differential cultivars); foxtail millet (Aka-awa); common millet (collected in Japan); *Digitaria sanguinalis* (purchased from Herbseed Co., UK); Italian ryegrass (Ace).

Pyriculol

Epipyriculol

Tenuazonic acid

Pyriculariol

Pyricuol

Pyrichalasin H

3-(1',3'-pentadienyl)-3,4-dihydro -1*H*-2-benzopyran-4,8-diol

4-(1'-hydroxy-2'-butenyl)-1,4 -dihydro-2,3-benzodioxocin-10-ol

Fig. 1 Phytotoxins produced in cultures of *Pyricularia* isolates

filtering the cultures through filter paper. The filtrate was passed through a Sep-Pak C_{18} cartridge (Waters) and eluted with methanol. The methanol eluate was analyzed by HPLC [column, Shim-Pack CLC-ODS (M) (4.6 mm I.D. × 250 mm); MeOH:water (7:3, V/V); flow rate, 1 mL/min; detection, A_{215}] and the quantity of pyrichalasin H was determined with the standard curve produced using the authentic compound.

2.3 Sporulation of P. isolates and Inoculation with Their Conidia on Digitaria

These details were described by Tsurushima et al. (2005).

2.4 Plant Material (Wheat)

Wheat (*Triticum aestivum* Thell.) cv. Norin 4 was bioassayed for necrosis-inducing activity in each fraction from the culture of a *Triticum* isolate, Br48. Wheat seeds were sown in vermiculite and grown in a growth chamber at 20 °C under 10,000 lux fluorescent light for a 16-hr period. The primary leaves of 14-day-old seedlings were used.

2.5 Bioassay of Necrosis-Inducing Activity on Leaf Segments

Each fraction from the culture of a *Triticum* isolate was dried using an evaporator and dissolved in a small quantity of a 1% ethyl alcohol solution. Individual leaf segments (4 mm wide) of wheat were incubated with 150 μL of the solution in the wells of a 96-well microplate (Falcon, Well dia. 6 mm). The microplate was incubated at 23 °C in light (400 W, continuous irradiation). The necrosis-inducing activity on the leaf segments was observed 36 h after the treatment.

3 Results and Discussion

3.1 Pathogenicity Determination and Chemotaxonomy of Pyricularia Isolates by Pyrichalasin H

Pyricularia grisea was originally identified in 1880, as causing gray leaf spots on crabgrass (Saccardo 1880). *Pyricularia oryzae* was identified as causing gray leaf spot on rice (Cavara 1891). Since then, the names *P. oryzae* and *P. grisea* have been applied to isolates from rice and from crabgrass (*Digitaria sanguinalis*), respectively. The teleomorphic states of these two species have not been found in nature. Hebert (1971) described the teleomorph *Ceratosphaeria grisea* based on an *in vitro* cross between isolates of the anamorph, *P. grisea*, from crabgrass. The teleomorph was changed as *Magnaporthe grisea* (Barr 1977; Yaegashi and Udagawa 1978). However, based on overlap in conidial morphology and interfertility among isolates from rice and other grasses, *P. oryzae* was synchronized under *Pyricularia grisea* Sacc. [teleomorph: *Magnaporthe grisea* M. E. Barr] (Rossman et al. 1990). This idea has become established among blast researchers around the world in the 90s as a main concept about species of *Pyricularia* isolates.

However, new molecular approaches applied to blast fungi in the early 2000s have broken down the concept of one species among *Pyricularia* isolates on gramineous plants (Kato et al. 2000; Couch and Kohn, 2002). Based on pathogenicity, mating ability and restriction fragment length polymorphisms with single copy DNA probes, 85 isolates of *Pyricularia* collected from 28 host plants have been classified as two different species, *Pyricularia oryzae* and *P. grisea*. These two species were further divided into seven pathotypes: the finger millet, foxtail millet, common millet, rice, crabgrass, Italian ryegrass/weeping lovegrass, and non-cereal/grass types (Kato et al. 2000). Phylogenetic trees of *Pyricularia* isolates were built up by comparing partial sequence of three genes: actin, beta-tubulin, and calmodulin (Couch and Kohn 2002). As a result, *Magnaporthe* isolates on rice and other cultivated grasses were described as *M. oryzae*, distinct from *M. grisea*, the specimen type of which was isolated from the genus *Digitaria*. However, this controversy about the species of *Pyricularia* isolates on crabgrass and rice still existed in this 4th International Rice Blast Conference.

Table 2 Pyrichalasin H production and pathogenicity on *Digitaria* of *Pyricularia* isolates

Numbers of *Pyricularia* isolates	Host of *Pyricularia* isolates	Production of Pyrichalasin H[a]	Pathogenicity on *Digitaria*[b]
32	rice, Italian ryegrass, foxtail millet, common millet green bristlegrass, finger millet, wheat, Lehmann lovegrass, knotroot bristlegrass, jungle rice rice cutgrass, buffelgrass, bamboo grass, etc.	0	0
32	*Digitalia sanguinalis* (crabgrass)	31	31
1	*Digitaria smutsii* (pangola grass)	1	1
7	*Digitaria holizontalis*	6	6
Total 72		38	38

[a] Numbers of *Pyricularia* isolates that produced pyrichalasin H.
[b] Numbers of *Pyricularia* isolates that showed pathogenicity on *Digitaria*.

Pyrichalasin H, first isolated from a *Digitaria* isolate was analyzed in 72 *Pyricularia* isolates from various gramineous plants using HPLC (Tsurushima et al. 2005). There is a correlation between pyrichalasin H production and the ability of *Pyricularia* isolates to infect *Digitaria* plants (Table 2). Pretreatment of leaf sheaths of crabgrass with pyricalasin H led to penetration and colonization by nonhost isolates. Thus, we proposed that pyrichalasin H might be responsible for the specific pathogenicity of *Pyricularia* isolates on the *Digitaria* genus. Our data also supported the concept that *Pyricularia* isolates infecting *Digitaria* were a different species from *Pyricularia* isolates infecting rice and the cereals.

3.2 Necrosis Inducers from Pyricularia Isolates

We searched for the necrosis-inducing factor in cultures of a *Triticum* isolate, Br48, because the isolate produces necrotic lesions on the leaves of wheat. The isolate was inoculated on 500 petri dishes (9 cm in diameter) containing oatmeal agar (1 L contains: oatmeal, 40 g; agar, 20 g; sucrose, 5 g; and water). After 10 days, these cultures were dipped in acetone. An acetone extract was evaporated to obtain an aqueous fraction, which was extracted with ethyl acetate. The extract was chromatographed on a silica gel column (Kieselgel 60, 230-400 mesh ASTM, E. Merck) by stepwise gradient elution (chloroform-MeOH, from 100:0, 99:1, 97:3, and 95:5 to 9:1). Five hundred-milliliter fractions were collected and the active principles were detected in the fractions eluted with chloroform containing 3% MeOH. The active fraction was purified with HPLC on an ODS column (flow rate: 0.8 mL/min, detector: RI) by eluting with MeOH-water (6:4, V/V). Fractions of two peaks (retention times: 11 min and 12 min) showed the necrosis-inducing activity on leaf segments of wheat (Fig. 2). Their yields were 11 mg and 14 mg respectively. Their molecular weights

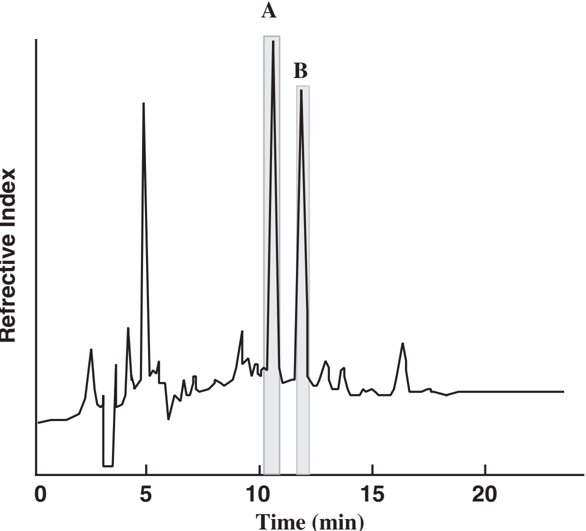

Fig. 2 HPLC profile of the extract from culture of Br48 isolate. The extract from the culture filtrate was analyzed by HPLC [column, Shim-pack PREP-ODS (H) Kit, 4.6 mm I. D. × 250 mm; MeOH-water (6:4, V/V); flow rate, 0.8 mL/min]. Detector: RI. A and B show pyriculol and epipyriculol, respectively

were estimated by EI-Mass to be both 248. They were identified as pyriculol (1) and epipyriculol (2) by comparing with the spectrum of the authentic pyriculol using mass spectrometry and NMR. Dihydropyriculol, a non-active pyriculol-related compound, was also detected in the same cultures and the yield of this compound was 32 mg. We also examined these two toxins in extracts from a *Triticum* isolate (Br3), an *Avena* isolate (Br58) from oat (*Avena sativa*), two *Setaria* isolates (GFSI1-7-2, NRSI2-2-2) from foxtail millet (*Setaria italica*), two *Eleusine* isolates (G10-1, Z2-1) from finger millet (*Eleusine coracana*), two *Digitaria* isolates (IBDS5-1-1, NI980), and two *Panicum* isolates (NNPM1-2-1, STPM1-3-2) from common millet (*Panicum miliaceum)*. The two toxins were detected in all extracts.

Br48 was also cultured in 500-mL Erlenmeyer flasks containing 100 mL of a soy sauce-sucrose medium (1 L containing: soy sauce, 50 mL; sucrose, 50 g; and water) by shaking (110 r.p.m) at 25 °C. After 10 days, the culture filtrate (total volume of 2 L) was extracted with ethyl acetate. The ethyl acetate extract was chromatographed on a silica gel column (Kieselgel 60) by stepwise gradient elution (chloroform-MeOH system). The active principles were detected in the fractions eluted with chloroform containing 3% MeOH. The active fraction was purified with HPLC on the ODS column. Pyriculol (1 mg) and epipyriculol (0.5 mg) were isolated as active principles. From these results, we determined that these two compounds might be the main necrosis-inducing principles of the blast fungus. Nukina (1998) described that all *Pyricularia* isolates from gramineous plants produced dihydropyriculol but some of them did not produce pyriculol. This difference in pyriculol production might be caused by the stock condition of *Pyricularia* isolates. Nukina has used *Pyricularia* isolates on cultures that were received from various institutes in Japan (from personal communication with Dr. Nukina). We used *Pyricularia* isolates that were stored on sterilized barley seeds as stock cultures in Kobe University. When

we examined the pathogenicity of *Pyricularia* isolates kept on PDA, several isolates showed reduced pathogenicity on their original host plants after being in the storage for three months (data not shown). Some isolates had completely lost their pathogenicity on PDA storage for six months. The isolates that lost their pathogenicity did not produce any peaks containing pyriculol on HPLC analyses (data not shown). However the pathogenicity of *Pyricularia* isolates has been unchanged on stock cultures of barley seeds over several years. Pyriculol and epipyriculol might act as factors related with disease symptoms of blast fungus although it should be determined whether *Pyricularia* isolates secrete the sufficient amounts of these toxins at their infection sites in host plants.

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An Unprecedented Outbreak of Rice Blast on a Newly Released Cultivar BRS Colosso in Brazil

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Abstract Rice blast occurred in epidemic proportions on a newly released upland rice cultivar BRS Colosso, in the rice growing season 2004/2005. Twenty isolates were collected from the affected panicles of the cultivar BRS Colosso from three different States, two pathotypes IB-1 and IB-17 were identified. They were classified into thirteen Brazilian pathotypes based on the reaction pattern on eight upland rice cultivars, utilized as local differentials. Differences in aggressiveness of the isolates on the cultivar BRS Colosso were evident. Ten highly aggressive isolates were used to determine the partial resistance to leaf blast in the BRS Colosso and BRS Bonança. There was no significant isolate x cultivar interaction for partial resistance. The mean leaf blast severity was significantly higher in BRS Colosso than in BRS Bonança. Inoculation of culms with the same ten isolates showed cultivar x isolate interaction. Some isolates were more aggressive showing severe culm blast. There was no correlation among the aggressiveness of the isolates on leaf and culm. The disease outbreak in BRS Colosso could be attributed to the absence of adequate degree of partial resistance, the preexistence of compatible pathotypes IB-1 and IB-17 and their dissemination through seed.

Keywords Epidemiology · *Magnaporthe oryzae* · Partial resistance

1 Introduction

An unprecedented rice blast outbreak caused by *Magnaporthe oryzae* [(Hebert) Yaegashi & Udagawa)] (Couch and Kohn 2002) [anamorph *Pyricularia grisea* (Cooke) Saccardo] has been reported in the rice growing season 2004/2005, in different commercial farms of a newly released cultivar BRS Colosso, by rice producers and confirmed by various research scientists and extension agents during their visits. The upland rice cultivar BRS Colosso developed for superior long grain

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quality and high yield potential was released for cultivation during 2003/2004 growing season. It was grown in extensive areas in the States of Minas Gerais, Goiás, Tocantins and Mato Grosso. The losses were found to be high and some farmers could not even harvest a single bag of rice. It is difficult to define the point at which an outbreak of a disease can be considered an epidemic. The widely cited disease epidemics such as coffee rust, maize rust, soybean rust are those which were destructive covering large areas in a short period of time. There are several epidemics which are local due to differences in climatic, topography and soil conditions. It is not necessary that all epidemics cover large areas but there may be destructive outbreaks over limited areas or limited to one cultivar. The only common factor is the destructiveness and loss in grain yield, but there is no numerical threshold or value at which an outbreak deemed to become an epidemic (Tarr 1972).

The affected plants showed typical blast symptoms on internodes and nodes as well as neck and panicle blast. The widespread occurrence of nodal blast in the upland rice may be considered as the first report even though it is commonly observed in irrigated rice. The panicle blast could not be controlled even with two applications of a systemic fungicide. The destructive nature of rice blast has been known world wide (Ou 1985) but the total grain yield losses even in the first year of release of this new cultivar lead to different speculations as to its cause. It was attributed to high blast susceptibility of the cultivar, intensive cultivation using heavy doses of nitrogen fertilizer, late application of nitrogen top dressing, favorable weather conditions, drought stress before or soon after panicle emergence, occurrence of a new race of pathogen or a high frequency of the preexisting matching races to the resistance genes of this cultivar, vertifolia effect due to lack of partial resistance, seed-borne infection, the resistance of the pathogen to the widely used fungicide tricyclazole.

Other cultivars, such as BRS Primavera and BRS Bonança, in the same farm or neighboring rice farms were found to be affected, but did not cause as colossal yield losses. Because of this observation the weather factor favorable to the occurrence of disease in epidemic proportion was ruled out. The cultivar has been tested in yield trials and blast nursery over a period of three years before its release. The examination of disease scores in the field trials showed that the panicle blast scores were low or similar to the susceptible checks. The mean leaf blast rating in blast nursery was 3.5 with a score ranging 0–5 in eight different test sites, whereas the susceptible cultivar Primavera showed mean score of 4.5 and 0–9 range. The data showed that the cultivar BRS Colosso exhibited susceptible reaction for leaf blast in tests before its release, but did not fully explain the sudden blast outbreak of this cultivar in the first year of its release. In general, the blast susceptibility of a given cultivar increases with an increase in area cultivated as a result of increase of a certain pathotype (Kiyosawa and Shiyomi 1976) in a stepwise manner. The short lived nature of varietal resistance is commonly attributed to mutations that change the phenotype of a strain from avirulence to virulence, sexual recombination, asexual recombination, acreage of resistant varieties, genetic uniformity of varieties and low field resistance (Kiyosawa 1982; Valent and Chumley 1994) and the failure of breeding lines to encounter low frequency compatible pathotypes in a population

(Correa-Victoria and Zeigler 1993) The widespread occurrence of rice blast on BRS Colosso, a year after its release required investigations as to the cause of the disease outbreak in order to adopt appropriate measures in the release of improved cultivars in future.

The present investigation reports the occurrence of blast disease outbreak on a newly released cultivar, the identification of pathotypes collected from the infected panicles of rice cultivar BRS Colosso from three States and the degree of partial resistance of the BRS Colosso in relation to the cultivar BRS Bonança.

2 Material and Methods

Single conidial isolates of *M. oryzae* were obtained from sporulating lesions on panicle branches of improved upland rice cultivar, BRS Colosso. Diseased samples were collected from farmers and experimental fields during two consecutive years for establishing isolates. Twenty isolates obtained from affected panicles were tested for identification of pathotypes and agressiveness of the virulent isolates.

Pathotypes were identified based on the reaction pattern on eight standard international differential cultivars (Dular, Kanto 51, NP125, Raminad Str 3, Usen, Zenith, Caloro and Sha-tiao-tsao) using the standard procedure (Atkins et al. 1967; Ling and Ou 1969) and eight Brazilian differential cultivars (Carajás, Confiança, Maravilha, Primavera, Progresso, Caiapó, IAC 47 and IAC 201) according to Prabhu et al. (2002a). Plant materials for tests were planted in plastic trays (15×30 cm) containing 3 kg of soil and fertilized with NPK (5 g of 5-30-15 + Zn and 3 g of ammonium sulfate per 3 kg of soil). Eight standard international and eight Brazilian differential cultivars were sown, in 5.0 cm long rows, in one tray for identifying pathotypes of the test isolates. Leaf blast reaction was assessed at seven to nine days after inoculation taking into consideration only two types of reaction of the host, compatible or susceptible and incompatible or resistant reaction. To determine the partial resistance, the cultivars BRS Colosso (Kay Bonnet/CNA 7119) and BRS Bonança (CT7244-9-2-1-52-1/CT7232-5-3-7-2-1P//CT6196-33-11-1-3-AP) were seeded in separate trays, in eight 10 cm long rows per tray.

Initially differences in aggressiveness among the isolates of *M. oryzae* were determined based on inoculation of BRS Colosso using 20 isolates and two replications. In another experiment the partial resistance in cultivars BRS Colosso and BRS Bonança was studied with 10 of 20 aggressive isolates using a split plot design with two replications. The main plots consisted of isolates and the subplots cultivars, in total two trays per isolate and replication. Inoculations were done on 21-day-old plants with spore suspension (3×10^5 conidia/mL) in greenhouse as described earlier by Araújo et al. (2005). A tray containing international differential cultivars as a non-inoculated control was maintained to ensure that no contamination occurred during the inoculation procedure. Inoculations were repeated twice and the one that gave consistent reaction was used for pathotype determination.

The percentage of leaf area infected was measured at nine days after inoculation, using a 10 grade visual rating scale, based on percentage of leaf area affected according to Notteghem (1981). A sample of 25 plants per replicate selected at random and the top fully opened leaf per plant was used for leaf blast assessment. For the variance analysis the disease severity data were transformed to arcsin $\sqrt{x}$.

For determining the partial resistance to culm blast of the cultivar BRS Colosso in relation to the cultivar BRS Bonança, the two cultivars were seeded in plastic pots containing 5 kg of soil fertilized with NPK in greenhouse. A completely randomized block design with three replications was used. Inoculations were done using the injection method with ten isolates of pathotypes IB-1 and IB-17. Inoculations were done soon after panicle emergence when the distance between the collor of the flag leaf and panicle base was approximately 3–4 cm length. Spore suspension (0.05 ml/culm) was injected into the uppermost internode 2.0 cm below the panicle base. Three tillers/plant in total of nine tillers in 3 replications were inoculated. Disease assessment was done at twelve days after the inoculation of culms with spore suspension. Lesion size from the point of inoculation was used as criterion for assessing partial resistance because of the differences in spikelet sterility among inoculated tillers within the same plant.

3 Results and Discussion

Initial inoculation tests with 20 isolates collected from the affected panicles of the cultivar BRS Colosso showed differential interaction for resistance to leaf blast. However, all isolates showed susceptible reaction on BRS Colosso and the cultivar Liderança which was developed from its sister line of the same cross. None of the test cultivars showed resistance to all isolates (Table 1). Out of 20 isolates of *M. oryzae* collected from BRS Colosso during the epidemic year 2005 and the following year 2006, 14 were identified as belonging to pathotype IB-1 and six as IB-17 based on the reaction pattern of eight international differentials (Table 2). These pathotypes were found to be distributed independent of collection site, State and year of collection. The predominance of the pathotypes IB-1 was also reported in earlier tests conducted during 1986-88, among isolates retrieved from upland rice cultivars (Prabhu et al. 2002a). The pathotype IB-17 was reported to occur in the cultivar Liderança (CNAs 8983) in a previous investigation (Prabhu et al. 2003). New races of the pathogen may be introduced from elsewhere through seed or long distance dissemination of spores by wind. Sometimes the appearance of a new race with new parasitic potentialities through variation producing mechanisms such as genetic mutation and parasexuality may result in disease in epidemic proportions. The destructiveness may be enhanced by widespread cultivation of a susceptible cultivar which has not previously encountered the particular race present in the area. The results showed that these pathotypes were pre-existing and IB-1 was one of the predominant pathotypes on upland cultivars. Occasionally, a preexisting race or pathotype in low frequency increases in high proportion with the release of a new cultivar. The rice blast outbreak on irrigated rice cultivar Epagri 108 and

Table 1 Reaction of 12 commercial upland rice cultivars to *Magnaporthe oryzae* isolates retrieved from BRS Colosso

	Upland rice cultivars											
Isolate	1	2	3	4	5	6	7	8	9	10	11	12
Py-8762	R[1]	R	S[2]	S	R	R	S	S	R	R	S	S
Py-8767	R	S	S	S	R	S	S	S	R	S	S	R
Py-8769	R	S	S	S	R	S	S	S	R	S	R	S
Py-8770	R	R	R	R	R	S	S	S	R	S	S	S
Py-8784	R	S	S	S	R	R	S	S	S	S	S	S
Py-8788	R	R	S	R	R	R	S	S	S	S	S	S
Py-8790	R	S	S	S	R	S	S	S	R	S	S	S
Py-8793	R	R	R	R	R	R	S	S	R	R	S	S
Py-8794	R	S	S	S	R	R	S	S	R	R	S	R
Py-8796	R	S	S	R	R	R	S	S	S	R	R	R
Py-8797	R	R	S	R	R	S	S	S	R	S	S	R
Py-8798	R	R	S	S	R	S	S	S	R	S	S	S
Py-8803	R	S	S	S	R	S	S	S	R	R	S	R
Py-8806	R	R	S	S	R	R	S	S	R	S	S	S
Py-8807	R	R	R	R	R	R	S	S	R	S	S	S
Py-8812	S	S	S	S	S	R	S	S	R	S	S	S
Py-8815	R	R	S	S	R	R	S	S	R	S	S	S
Py-8816	R	S	S	S	R	S	S	S	R	S	S	R
Py-8818	R	R	S	R	R	S	S	S	R	S	S	R
Py-8821	R	S	S	S	R	R	S	S	S	S	S	S

[1] 1-Carajás; 2-Confiança; 3- Maravilha; 4-Primavera; 5-Caiapó; 6-Progresso; 7-Colosso; 8-Liderança; 9- Curinga; 10-Conai; 11-Vencedora, 12-Bonança.
[2] R = Resistant; S = Susceptible

109, one year after their introduction in the State of Tocantins was attributed to the increase of pathotype IB-45, which was possibly existing in a low frequency (Prabhu et al. 2002b). One of the possible explanations for the high frequency of pathotypes IB-1 and IB-17 in different and distant localities can be attributed to the primary source of inoculum originating from the infected seed. The basic seed was multiplied originally by three seed producers, in three different localities in the State of Mato Grosso during 2003/2004 rice growing season. Three hundred and thirty eight tons of seed, produced in an area in total 190 hectares was sold for planting about 5000.00 hectares in the following year 2004/2005. Comparison of pathotypes collected during the epidemic year and the succeeding year showed similar distribution pattern.

Thirteen pathotypes were identified based on Brazilian differential cultivars in contrast to two international pathotypes IB-1 and IB-17. They represented two race groups BB and BC. The differential cultivars Maravilha, Primavera, Progresso, IAC 201, Confiança, IAC 47 and Caiapó in descending order showed susceptible reaction to the large number of isolates tested. None of the 20 isolates of *M. oryzae* obtained from BRS Colosso were compatible to the differential cultivar Carajás. The reaction pattern on local Brazilian differentials furnish information on resistance gene frequencies that are useful for breeding purpose.

The analysis of variance of the data on percentage leaf area affected, in inoculation test of cultivar BRS Colosso with 20 isolates, showed significant differences

Table 2 Isolates of *Magnaporthe oryzae* collected from affected panicles of the cultivar BRS Colosso, pathotypes and percentage leaf area of cultivar BRS Colosso in greenhouse inoculation tests

Isolate[1]	Collectionsite/State	IP[2]	BP[3]	Diseased leaf area (%)
Py 9057-P1[1]-06*[5]	Faz.Capivara/GO	IB-1	BB-16	59.82 a[4]
Py 8790-P2[1]-05*	São Bernardo/GO	IB-1	BB-2	52.70 a
Py 9056-P4[1]-06*	Lagoade Confusão/TO	IB-1	BB-8	52.56 a
Py 8806-P3[1]-05*	Piu/TO	IB-1	BC-16	52.20 a
Py 9061-P2[1]-06*	Faz.Capivara/GO	IB-17	BC-7	49.90 ab
Py 8766-P2[1]-05*	Agua Boa/MT	IB-1	BB-7	49.26 ab
Py 9063-P4[1]-06*	Faz.Capivara/GO	IB-17	BC-7	48.12 ab
Py 8800-P2[1]-05*	Uruana/GO	IB-1	BC-5	47.16 ab
Py 9052-P1[1]-06*	Lagoa de Confusão/TO	IB-1	BB-7	46.72 ab
Py 9053-P2[1]-06*	Lagoa de Confusão/TO	IB-1	BB-8	41.95 abc
Py 9059-P3[1]-06	Faz.Capivara/GO	IB-17	BC-32	34.10 abcd
Py 8813-P2[3]-05	Faz.Capivara/GO	IB-1	BB-5	34.00 abcd
Py 8812-P1[2]-05	Faz.Capivara/GO	IB-17	BC-7	27.84 abcd
Py 8804-P2[1]-05	Piu/TO	IB-17	BC-16	27.74 abcd
Py 8793-P3[1]-05	São Bernardo/GO	IB-1	BB-7	26.55 abcd
Py 8762-P1[1]-05	Agua Boa/MT	IB-1	BB-1	25.06 abcd
Py 8811-P1[1]-05	Faz.Capivara/GO	IB-17	BC-6	20.70 abcd
Py 9068-P1[1]-06	Faz.Capivara/GO	IB-1	BB-13	5.09 bcd
Py 8770-P1[1]-05	Agua Boa/MT	IB-1	BB-16	2.15 cd
Py 8789-P1[1]-05	São Bernardo/GO	IB-1	BC-24	0.78 d

[1] Py refers to *P. grisea* is followed by accession number of culture collection (Mycotec), *P* indicates panicle number, superscript refers to conidial number, the last two digits indicate year of collection; isolates followed by asterisks were used for partial resistance analysis of the cultivar BRS Colosso.

[2] Pathotypes identified using eight standard international differentials.

[3] Pathotypes identified using eight Brazilian differentials.

[4] Means disease severities followed by the capital letters in the line differ significantly, according to Tukey's test at the 0.05 probability.

[5] Isolates followed by asterisc were utilized for determining partial resistance of rice cultivars BRS Colosso and BRS Bonança.

in aggressiveness (Table 2). For disease to occur the pathogen must be virulent; it may be virulent and strongly aggressive or weakly aggressive (Van der Plank 1975). The results in the present study showed, 10 of the 20 isolates were significantly more aggressive on BRS Colosso, independent of location and year of collection. Differences in aggressiveness of isolates pertaining to the same pathotype IB-1 were evident in inoculation tests on leaf. The damage level of rice cultivars could change over time not only due to change of race but also due to change in aggressiveness of the blast isolate belonging to the same race or lineage. A significant increase in aggressiveness of blast isolates in the cultivar C101A51 possessing *Pi-2* gene was observed. On the other hand aggressiveness of isolates from known durably resistant cultivars IR 64 and IR 36 was much lower than C101A51(Ahn 2000).

The analysis of variance of data on percentage leaf area affected and lesion size on culms, in inoculation test using 10 isolates of *M. oryzae* and two cultivars BRS Colosso and BRS Bonança are presented in Table 3. There was a highly significant main effect indicating difference in partial resistance between cultivars. There

Table 3 Analysis of variance for leaf blast severity[1] of rice cultivars BRS Colosso and BRS Bonança inoculated with 10 isolates of *Magnaporthe oryzae*

Source	df	Sum of squares	Mean square	F value
Cultivar (C)	1	8398.40	8398.40	113.24*
Isolate (I)	9	188.73	20.97	0.28 ns
CxI	9	171.21	19.023	0.26 ns
Error	20	1483.34	74.167	
Total	39	10241.70		

[1] Disease severity data were transformed to arcsin $\sqrt{x}$ for analysis; *F value significant at 0.1% probability levels.

was no significant difference in aggressiveness among isolates, with nine degrees of freedom, as well as cultivar x isolate interaction (Table 3). These results are in accord with those obtained by Yeh and Bonman (1986) and Araújo and Prabhu (2002). The main effects and the first order interaction determine aggressiveness of the isolates/partial resistance and virulence/vertical resistance, respectively. The inoculation tests with *M. oryzae* showed no evidence for vertical resistance in the cultivars BRS Colosso and BRS Bonança, because all 10 isolates were virulent considering percentage leaf area affected. The mean percentage leaf area of BRS Colosso (50.03%) was significantly higher than that of BRS Bonança (8.59%), indicating absence of adequate degree of partial resistance to leaf blast. The exotic source of resistance such as Key Bonnet, utilized as one of the parents in the cross, possibly with no partial resistance, might have contributed to the high vulnerability of BRS Colosso to blast.

The analysis of data on culm blast showed significant isolate x cultivar interaction. Differences in lesion size on culm were significant (Table 4). A majority of the isolates on BRS Colosso were more aggressive than on BRS Bonança. Despite

Table 4 Leaf and culm blast severities inoculated with 10 isolates of *Magnaporthe oryzae* in greenhouse

	BRS Colosso		BRS Bonança	
Isolate	LBS(%)[1]	CBS[2]	LBS (%)	CBS
Py 9057-P1[1]-06	59.82[ns]	0.69 a[3]	6.82[ns]	0.31 b
Py 8790-P2[1]-05	52.65	0.76 a	15.74	0.52 ab
Py 9056-P4[1]-06	52.56	0.31 b	8.69	0.37 ab
Py 8806-P3[1]-05	52.20	0.40 a	10.56	0.21 b
Py 9061-P2[1]-06	49.90	0.57 a	3.60	0.77 a
Py 8766-P2[1]-05	49.26	0.56 a	4.58	0.15 b
Py 9063-P4[1]-06	48.12	0.54 a	13.91	0.21 b
Py 8800-P2[1]-05	47.16	0.39 a	6.22	0.51 ab
Py 9052-P1[1]-06	46.72	0.47 a	6.44	0.30 b
Py 9053-P2[1]-06	41.95	0.50 a	9.30	0.42 ab
Mean severity	50.035 A[4]	0.52	8.590 B	0.38

[1] Leaf blast severity; [2] Culm blast severity (disease index), [3] Letters followed by the same letter in the column do not differ significantly, according to Tukey's test (5%) ns = non-significant. [4] Means disease severities followed by the capital letters in the line differ significantly, according to Tukey's test at the 0.05 probability.

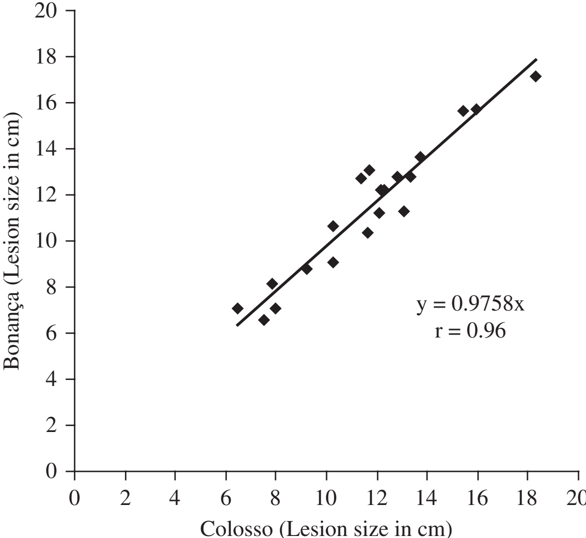

Fig. 1 Aggressiveness of field isolates of *Magnaporthe oryzae* on culms of cultivar BRS Colosso and BRS Bonança in inoculation tests using 19 isolates collected from affected panicles of BRS Colosso

differences in aggressiveness among isolates on these two cultivars, there was good correlation between lesion size of BRS Colosso and BRS Bonança in artificial inoculation test (Fig. 1). The interaction was of small magnitude. Significant interactions for partial resistance to leaf blast were reported in earlier studies (Bonman et al. 1989; Araújo and Prabhu 2002).

Occurrence of rice blast on widely grown susceptible cultivar such as BRS Bonança rarely attain damage levels similar to those of the cultivar BRS Colosso and is largely determined by agricultural practices such as late plantings and late application of N fertilizer as top dressing.

Leaf blast was not reported to occur in serious proportions on BRS Colosso both in early and late plantings. Leaf blast epidemics are characterized by a rapid increase of a disease to a distinct peak followed by decline, as the plant become more resistant with age. However, conidia from the infected leaves serve as inoculum for panicle infection. The increase in conidial production is exponential until the panicle emergence, independent of blast disease severity on leaves. The lack of inoculum or differences in quantity of inoculum is not a factor to be considered. The pathogen has sufficient time to multiply on leaves, abundant quantities of inoculum required for the panicle infection. Epidemics are likely to break out when a susceptible crop of BRS Colosso is exposed to abundant viable inoculum of a virulent pathogen originating from seed, under environmental conditions favorable for panicle infection. The role of infected rice seed as primary source of inoculum for initiating leaf blast epidemics in irrigated rice has been well documented (Lee 1994)

When both cultivars BRS Colosso and BRS Bonança are susceptible to the same pathotypes of the pathogen as is evident in the present study, a disease outbreak in one cultivar is expected to give rise to secondary outbreak in the cultivar planted at a later date. The infected plants of the earlier sown cultivar provide massive quantities

of inoculum for the later sown cultivar and is likely to be more severely attacked. But, blast disease outbreak similar to that observed on BRS Colosso have not been reported on BRS Bonança in farmers' fields, even in late plantings. In experimental fields of Embrapa Rice and Bean Research Center, when both BRS Bonança and BRS Colosso were planted in adjoining plots, the disease was destructive only on BRS Colosso but not on BRS Bonança which can be attributed differences in the degree of partial resistance to blast, in addition to the seed borne inoculum of the pre-existing pathotypes.

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Effect of *Magnaporthe grisea* on Seed Germination, Yield and Quality of Wheat

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Abstract The effect of wheat blast, caused by *Magnaporthe grisea* (*Pyricularia grisea*), on seed germination, yield loss and grain quality was evaluated during the growing season of 2005 in wheat cultivars BRS208 and CD104 in Sao Paulo State, Brazil. Each field was divided in an imaginary diagonal line crossing the center and then five areas of one m^2 each separated 10 m were selected. Cultivars differed significantly in their reaction towards blast. The BRS208 showed to be more susceptible with incidence of 76.2%, higher severity and yield loss of 662.2 kg/ha or 32.2% whereas CD104 had lower incidence (32%), severity and yield loss (399.1 kg/ha or 13.9%). Although a clear difference was detected between weight of 100 seeds from healthy-looking and blast diseased heads there was no difference in seed infection, which ranged from 68.6 to 83.1% in these two cultivars. Moreover there was no correlation between seed infection and germination in any cultivar. Seeds from blast diseased heads had higher proteins contents whereas lipids remained unaffected. Finally it is worth stressing that incidence of disease, yield loss and other parameters evaluated in this work were carried out in a commercial field with two applications of fungicides. The present data showed the inefficacy of chemicals to control wheat blast and that the disease continues to be one major problem of wheat in Brazil.

Keywords *Pyricularia grisea* · Wheat blast · Yield loss

1 Introduction

Magnaporthe grisea (Hebert) Barr (anam. *Pyricularia grisea* Sacc.), the causal agent of blast disease, is a pathogen that attacks more than 50 grass species (Ou 1985). Rice (*Oryza sativa* L.) is the most important crop for which severe yield loss

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has been reported worldwide. In Brazil, wheat (*Triticum aestivum* L.) is another cereal that shows high incidence of fungal infection. It was first reported in 1986 in northern cities of Parana state, the Brazilian most important producer (Igarashi et al. 1986) and spread subsequently to other states of the Country (Anjos et al. 1996; Goulart et al. 1990; Igarashi 1990; Picinini and Fernandes 1990; Prabhu et al. 1992). It is currently one major disease of wheat in Brazil due to its widespread distribution, lack of efficient fungicides and effective resistant varieties and significant yield loss (Goulart and Paiva 2000; Igarashi 1990; Urashima and Kato 1994; Urashima et al. 2004).

The most conspicuous symptom of wheat blast in the field is the head infection. The region above the infection point gets bleached and straw colored and the uptake of nutrients is hampered, as a consequence the affected spike can be clearly distinguished from the healthy one. Depending on the infection site in the spike and the plant stage, severe yield loss can be inflicted because grain formation above the infection point can be nil. Leaf infection is seen only in highly susceptible cultivars.

Despite the frequent occurrence of the disease in many regions of Brazil, updated information on yield loss is rare. In the past, an assay detected incidence of head blast ranging from 48 to 93% and yield loss varying from 10.5 to 40% during 1988–1990 wheat growing seasons (Goulart and Paiva 1992). In the following two years, an average of 86% of incidence with 51% of yield loss was observed (Goulart and Paiva 2000). Both studies employed Anahuac, a variety of Mexican origin, very popular among Brazilian farmers in early 90s but no longer cultivated due to its high susceptibility to blast.

The effect of the presence of *M. grisea* on wheat seed germination is also little known in spite of reports on the presence of the pathogen on seeds (Lopes and Bueno 1990), its transmission to seedlings (Goulart and Paiva 1991), its survival (Reis et al. 1995), and chemical control (Goulart and Paiva 1993).

No study is available on the effect of the pathogen on the nutritional quality of damaged grains. Therefore, the present work aimed to examine the effect of *M. grisea* on seed, yield and nutritional quality of two wheat cultivars currently grown in Brazil.

2 Material and Methods

2.1 Host and Sampling Strategy

This work employed wheat cultivars BRS208 and CD104, two of the most popular in the state of Sao Paulo, during the wheat growing season of 2005. They were sown on the same day, side by side, and submitted to the same agronomic practices in the municipality of Itapeva. Two fungicide applications with mancozeb + methyl tiophanate were carried out in an attempt to suppress the adverse effect of head blast: first at blossoming stage and second three weeks later.

Sampling was carried out one week before harvest. Each field was divided in an imaginary diagonal line crossing the center. From this point, five areas of one

m^2 each, separated 10 m between samples, were selected and all heads collected, deposited in a paper bag, identified and taken to laboratory.

2.2 Laboratory Trials

Wheat heads taken to lab underwent a preliminary screening by counting and separating diseased heads from healthy-looking ones according to visual presence of blast symptom. Subsequently, all grains were manually collected and allowed to dry at room temperature for further laboratory assays. The following parameters were evaluated: total weight of grains (converted in kg/ha), weight of 100 grains, percentage of grain (seed) infected by blast, percentage of seed germination, and centesimal composition of grains from healthy-looking and diseased heads.

2.3 Disease Assessment

The disease was evaluated by assessing the incidence and severity of blast on wheat heads. The incidence was calculated by estimating the total number of diseased heads. And then, a finer screening was performed by estimating the percentage of affected area: 0, 5, 25, 50, 75, and 100% of diseased heads, corresponding to the grades: 0, 1, 2, 3, 4, and 5, respectively (Faria et al. 1982). The severity of the disease (I.S.B.E.) was calculated as follow:

$$\text{I.S.B.E.} = \sum \text{class grade} \times \text{frequency/number of total heads}$$

d) Percentage of blast infection on wheat seeds

Infection by *M. grisea* was examined in grains from healthy-looking and diseased heads. This was examined employing blotter test in five replications in a randomized block design. Each replication consisted of fifty seeds of each variety uniformly distributed in a glass Petri dish with two of layers of paper filters. These papers were wetted with distilled water and kept at 25 C with photoperiod of 12 h for three days. The presence of *Pyricularia* spores were checked in a standard laboratory microscope.

2.4 Yield Loss Assessment

The method to assess yield loss in this study was similar to former works (Goulart and Paiva 2000; Goulart et al. 1992).

Considering,
Potential yield (PY) (g/m^2)
Total weight of grains from healthy heads/m^2 (TWHH)
Total number of healthy heads/m^2 (THH)
Total number of heads/m^2 (TH)
PY = (TWHH/THH) X (TH)
Actual yield (AY) (g/m^2)

Total weight of diseased heads/m^2 (TWDH)
$AY = TWHH + TWDH$

$\text{Yield loss} = PY - AY\ (g/m^2)$

2.5 Weight of 100 Seeds

For this parameter, five samples of wheat heads were first separated in two groups: heads with blast symptoms from those with no visual symptoms. From each group, 100 seeds were randomly collected and their weight measured employing an analytical scale with sensitivity on the order of micrograms.

2.6 Seed Germination

Germination of seeds was analyzed employing 25 seeds divided according to sanitary conditions of heads per replication distributed in a completely randomized design. A total of five replications were used. Glass Petri dishes with two layers of wetted paper filter accommodated the seeds and subsequently kept in 25 C with photoperiod of 12 h for four days. After this period the number of germinated seeds was visually determined.

2.7 Quality of Grains

This was studied through the centesimal composition utilizing grains separated according to sanitary condition of heads. To determine the lipids contents the method by Bligh and Dyer (1959) was applied and for proteins, humidity and ashes the method of the Association of Official Analytical Chemists (AOAC 1997).

2.8 Statistical Analysis

Data of incidence, severity and yield were analyzed by ANOVA. Because of high coefficient of variation, raw data of yield were transformed to $\sqrt{x}+0.5$ prior to analyses. Subsequently, the Tukey's at 5% probability was applied. Data on yield loss were submitted to Studentized range test at 5% probability. Centesimal composition data were analyzed by standard deviation of mean.

3 Results

Symptoms of leaf blast were not observed in any variety. The effect of *M. grisea* on heads of wheat cultivars BRS208 and CD104 is shown in Table 1. A striking difference in disease reaction was observed between these two cultivars. The BRS208

Table 1 Incidence, severity and yield loss in two wheat cultivars infected by *Magnaporthe grisea*

Cultivar	Total number of heads	Incidence (%)	Severity (ISBE[1])	Yield (Kg/ha)	Potential yield[2] (Kg/ha)	Yield loss3 (kg/ha)	(%)
BRS208	414.4	76.2[a*]	3.1634[a]	1391.60[a]	2053.80	662.20[a]	32.24[a]
CD104	380.6	32.0[b]	1.1937[b]	2476.30[b]	2875.40	399.10[b]	13.88[b]
C.V.(%)	N.A.	18.0	22.7	23.4	N.A.	N.A.	N.A.

[1] ISBE = sum of class values X frequency/total number of observations
[2] Potential yield (g/m^2) = [(Weight of grains from healthy heads/m^2)/(Number of healthy heads/m^2)] X total number of heads/m^2
[3] Yield loss (g/m^2) = Potential yield – (Weight of grains from healthy heads + Weight of grains from diseased heads)
* numbers followed by the same letter are not significantly different at 5% level
N.A. = not applicable

presented significant higher disease incidence (76.2%) comprising an average of 315.7 heads with blast symptom whereas CD104 had 32.0% which corresponded to 121.8 heads. The higher disease susceptibility of BRS208 was also visible when severity was examined. The ISBE index, that takes into consideration the number and area of head damaged, showed statistic different value between BRS208 (3.1634) and CD104 (1.1937). These results indicated that both higher number of heads and area affected by *M. grisea* in cultivar BRS208. Higher values of incidence and severity resulted in a statistic significant lower yield (1391.60 kg/ha) which also held true for the opposite: lower incidence and severity, significant higher yield (2476.30 kg/ha). Each cultivar has its own potential, the CD104 revealing to be more adapted to local conditions with potential yield ability up to 2875.40 kg/ha compared to 2053.80 of BRS208, had the disease not occurred. Consequently, wheat blast caused a yield loss of 662.20 kg/ha in the cultivar BRS208 (32.24%) whereas CD104 showed significant lower yield depletion of 399.10 kg/ha or 13.88%. In short, CD104 had a better performance than BRS208 towards blast disease when it was cultivated in a blast-prone region of Sao Paulo state because it showed lower degree of disease incidence, severity and yield loss with higher yield.

Further analysis between healthy-looking and diseased heads is shown in Table 2. Weight of 100 seeds from healthy-looking heads was significantly heavier regardless the cultivar when compared to seeds from blast diseased heads. There was

Table 2 Weight of 100 seeds, percentage of infection by *Magnaporthe grisea* and germination of seeds from healthy-looking and blast diseased heads in two wheat cultivars

Cultivar	BRS208 Healthy-looking	BRS208 Diseased	CD104 Healthy-looking	CD104 Diseased	C.V.(%)
Weight (g)	2.6775[p*]	1.8076[q]	2.7490[p]	2.0388[q]	13.8
Infection (%)	83.1[a]	77.1[a]	68.6[a]	71.8[a]	13.8
Germination (%)	62.4[x]	66.0[x]	34.0[y]	37.2[y]	24.8

* numbers followed by the same letter are not significantly different at 5% level

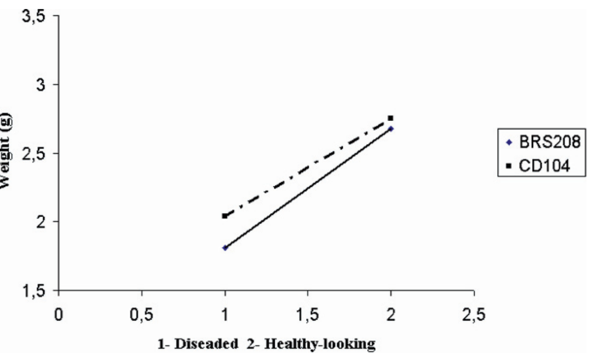

Fig. 1 Correlation between weight of 100 seeds from blast diseased and healthy-looking heads of two wheat cultivars

no statistic difference between cultivars regarding weight of seeds from healthy or diseased heads, ie, 100 seeds from health heads of BRS208 had no difference with seeds under the same condition from cultivar CD104. Figure 1 illustrates clearer that weight of 100 seeds collected from diseased heads from both cultivars was always lower than equal amount of seeds from healthy-looking heads. The visualization of seeds in Fig. 2 demonstrates that seeds differed in general aspect, size, color, etc, depending on sanitary condition of heads.

There was no difference of seed infection by *M. grisea* between healthy-looking and diseased heads in any cultivar (Table 2). Seeds from BRS208 had infection ranging from 77.1 and 83.1% and from 68.6 to 71.8% in CD104 showing that even in apparently healthy heads the pathogen was present in their seeds. When seed germination was examined significant difference was detected between cultivars, BRS208 had germination over 62% regardless of sanitary condition of the head, which was significantly different from CD104 with germination bellow 38%. Difference in germination was not observed between seeds from healthy-looking and diseased heads in the same cultivar.

As far as the nutritional composition of grains was concerned, both cultivars showed significantly higher proteins contents in grains from blast diseased heads than grains from healthy-looking heads (Table 3). When comparing two cultivars, this important element was significantly higher in BRS208. Difference in lipids and carbohydrates contents was not detected.

Fig. 2 Visual aspect of wheat seeds from healthy-looking (*left*) and blast diseased heads (*right*)

Table 3 Centesimal composition of wheat grains obtained from blast diseased and healthy-looking heads of two wheat cultivars

	BRS208		CD104	
	Diseased	Healthy-looking	Diseased	Healthy-looking
Lipids (%)	$2{,}60 \pm 0{,}12$	$2{,}56 \pm 0{,}17$	$2{,}58 \pm 0{,}18$	$2{,}57 \pm 0{,}18$
Ashes (%)	$1{,}93 \pm 0{,}06$	$1{,}83 \pm 0{,}06$	$1{,}76 \pm 0{,}06$	$1{,}56 \pm 0{,}06$
Proteins (%)	$15{,}02 \pm 0{,}82$	$13{,}33 \pm 0{,}66$	$12{,}97 \pm 0{,}13$	$11{,}46 \pm 0{,}38$
Humidity(%)	$12{,}43 \pm 0{,}31$	$12{,}06 \pm 0{,}11$	$12{,}33 \pm 0{,}06$	$11{,}81 \pm 0{,}15$
Carbohydrates (%)	68,20	70,22	70,36	72,60

4 Discussion

Since the first occurrence of wheat blast in some fields in northern Parana state in 1985, the most conspicuous symptom of wheat blast has been the head infection (Urashima and Kato 1994). All the following reports that described the spread of disease to other states of Brazil and Bolivia, noticed a severe yield loss because the disease occurred by the time of head emergency and grain formation (Barea and Toledo 1996; Goulart et al. 1990; Igarashi 1990; Picinini and Fernandes 1990; Prabhu et al. 1992). Seed contamination is suspected to be the main source for primary infection (Urashima et al. 1999). Survey carried out soon after the importance of wheat blast was observed revealed difference in disease reaction among cultivars but inexistence of resistance (Barros et al. 1989; Goulart and Paiva 1992; Igarashi 1990; Urashima and Kato 1994). Anahuac, one of the most important cultivars by the time of disease outbreak, exhibited yield loss ranging from 10.5 to 51% during 1988–1992, depending on region and weather (Goulart and Paiva 2000; Goulart et al. 1992).

Because of yield depletion of Anahuac and others with high susceptibility they were gradually been replaced. A study conducted many years later with 20 cultivars showed that cultivars extensive grown in Mato Grosso do Sul and Parana states at end of 1990's were still susceptible and none was resistant (Urashima et al. 2004). Besides incidence, other work done in 2004 with 20 other wheat cultivars showed yield damage ranging from 10.5 to 13% according to location and cultivar, stressing the continued importance of the disease (Goulart et al. 2007).

The present study demonstrated that *M. grisea* kept on causing severe damage even after 20 years of the first disease outbreak. Because resistant cultivar has been the most employed means to cope with blast disease, farmers are continuously looking for new cultivars. The BRS208, a cultivar released in 2001 as resistant to main foliar diseases of wheat and intermediate to blast (Embrapa 2007) had blast incidence as high as Anahuac grown in 1991/92 (Goulart and Paiva 2000) although yield loss was higher in the latter. Significant difference was also observed between BRS208 and CD104 in the present work revealing a clear difference in yield loss. Two reasons may be considered for this distinction: (a) the higher susceptibility of BRS208 to blast, (b) the difference in the time of heading. Although no information is available on the reaction of CD104 to blast, the BRS208 had one of the highest

disease incidence in a study carried out in Mato Grosso do Sul (Goulart et al. 2007). Besides, its heading occurs before CD104 (Coodetec 2007; Embrapa 2007). The fact that BRS208 has earlier heading may have great impact on yield loss since the earlier the heading the greater the chance for head infection because higher humidity is more prevalent in the early stages when compared to drier condition as the plant stage progresses (Goulart and Paiva 1992).

Yield loss up to 32.24% of BRS208, a cultivar extensively grown, indicates the need of searching for source of resistance to blast and other strategies to prevent such reduction. Unfortunately however, wheat blast has distinct features from the rice blast which make such task very difficult. Some reasons are: (a) there was no resistant cultivar to all isolates of *M. grisea* although they differed in degree of resistance (Urashima et al. 2004); (b) there was no association between resistance to leaf and head since cultivars resistant at seedling stage had high incidence of head blast (Arruda et al. 2005), which indicates that tests for resistance should be done at seedling as well as at reproductive stage; (c) *Brachiaria plantaginea* and *Digitaria insularis*, common weeds in wheat fields, may contribute to wheat blast because *M. grisea* from these hosts were capable of causing head blast (Urashima and Kato 1998; Urashima et al. 2003); (d) fungicides effective for rice blast were not capable to protect wheat heads (Goulart et al. 1996; Urashima and Kato 1994).

The absence of correlation between head blast symptom and seed infection was clearly visible, seeds collected from diseased heads had similar degree of infection of seeds from healthy-looking ones in both cultivars (Table 2). This result is in agreement with Jeyanandarajah and Seneviratne (1991) and Cornélio et al. (2000) in their study on rice blast pathosystem but distinct from Goulart et al. (1995) and Manandhar et al. (1998) where linear relationship was detected between neck blast symptoms and seed infection in wheat and rice blast diseases, respectively. One possible explanation for the discrepancy between those works and the present one could be the time of seed infection. The airborne inoculum may have reached seeds in the final stage of their formation and infection occurred only on the outer layer of seeds. Igarashi (1990) had already pointed out that with late infection grain fill will be normal but with higher chance of seed infection. Another data to support the hypothesis that infection occurred at later stage of seed formation comes from the germination index. Had the infection happened in the early stage of seed formation, the fungus would have caused the grains to be unfilled, preventing them from complete development (Manandhar et al. 1998).

The present work did not detect any deleterious effect of *M. grisea* on germination since seeds from diseased heads had similar germination of healthy-looking ones on both cultivars (Table 2). This finding did not corroborate Pande et al. (1994) where seeds of *Eleusine coracana* did not germinate because they were heavily colonized by *M. grisea*. One reason why germination of wheat was not affected despite the high seed infection may be the time of infection, as mentioned before. Infection at late stage of seed formation caused the pathogen to remain on the outer surface, invading only few cell layers of seed endosperm and germination is not affected because embryo remains pathogen-free (Chung and Lee 1983). Reason for the low germination of CD104 was not related to blast disease.

A straight correlation was identified between weight of 100 seeds from blast diseased and healthy-looking heads in both cultivars (Fig. 1). Seeds originated from diseased heads had a mix of smaller, lighter, and shriveled seeds with normal ones (Fig. 2).

The yield depletion and the low quality of grains observed in the present work are the main reasons to make blast a major disease of wheat in Brazil since its first outbreak. Differently from other foliar diseases of wheat, *M. grisea* characterizes for attacking mostly the producer organs, symptoms on leaves are rare. The degree of damage is directly correlated with time of infection, the earlier the greater (Igarashi 1990).

It is the first time the effect of blast disease on centesimal composition was examined. This was possible because seeds from diseased heads were collected manually. Usually in a commercial scale they are discarded during the post harvest process of threshing or winnowing. Although lipids contents were similar in both cultivars regardless of their sanitary condition, it was interesting to notice that proteins were higher in diseased grains. One explanation may be because the late infection of fungus which caused damage only on outer layers of seeds and did not reach the embryo cells of seeds that contains high contents of protein (Manandhar et al. 1998). This infection caused a reduction of seed size but not of protein and consequently increasing the percentage of it.

Finally it is worth stressing that incidence of disease, yield loss and other parameters evaluated in this work were carried out in a commercial field with two applications of fungicides. The yield loss observed here showed the poor efficacy of chemicals as in the past (Goulart et al. 1996; Urashima and Kato 1994) and that blast continues to be an important disease on wheat in Brazil.

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Part IV
Identification and Use of Resistance Genes and Breeding Strategies

Field Resistance Expressed when the *Pi-ta* Gene is Compromised by *Magnaporthe oryzae*

Fleet N. Lee, R.D. Cartwright, Yulin Jia and J.C. Correll

Abstract The *Pi-ta* gene provided 14 years of durable resistance to contemporary field population of *Magnaporthe oryzae* in southern USA rice production areas before being overcome during 2004 in 'Banks', a *Pi-ta*-based cultivar, by race IE-1k of the blast pathogen. Previously detected in production fields in 1994, the rarely recovered race IE-1k appeared to be poorly adapted to local conditions. Although stable molecular variations were defined in field isolates from 'Banks', virulence bioassays do not distinguish between isolates from Banks and the type race IE-1k isolate. After 2004, blast epidemics were noted in other 'Banks' production fields but have not been observed in other cultivars containing *Pi-ta* including 'Drew', 'Ahrent' and 'Cybonnet'. The *Pi-ta* allele in 'Banks' still confers resistance to all contemporary blast races except IE-1k and was determined to be molecularly identical to the *Pi-ta* allele in 'Katy'. 'Banks' does not contain the minor blast resistance genes *Pi-kh* or *Pi-ks* that are present in 'Drew', 'Ahrent' and 'Cybonnet'. An increase in leaf blast severity observed in moisture-stress tests using *Pi-ta* based cultivars suggests additional resistance genes, such the *Pi-kh* and *Pi-ks*, enhance *Pi-ta* gene efficacy against race IE-1k under field conditions. The data suggest that the *Pi-ta* gene functions as a partial resistance gene in 'Katy', 'Ahrent', 'Cybonnet', 'Drew', 'Banks', 'Tetep' and 'Tadukan' in regard to the broadly virulent blast pathogen races IE-1k and IB-33.

Keywords *Oryza sativa* · *Magnaporthe oryzae* · *Pi-ta* · *Pi-ks* · *Pi-kh* · Blast · Field resistance · Partial resistance

1 Introduction

Record of per hectare production of rice, *Oryza sativa* L. has been achieved due to large-scale grower adoption of high yield potential cultivars and modern production techniques in the southern USA (Wilson and Branson 2005; Wilson and

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Branson 2006). While many factors are responsible for the recent record-setting yield trends in southern rice, one very significant component is reliable control of rice blast disease caused by *Magnaporthe oryzae* [*Magnaporthe grisea* (Herbert) Borr. (anamorph *Pyricularia oryza* Cav.)] (C.E. Wilson, personal communication 2008; Lee et al. 2006). Blast disease control strategies in Arkansas and other parts of the southern USA include the use of R genes and flood-irrigation-induced field resistance (Marchetti 1994; Lee 1994; Lee et al. 2006).

Major blast resistance genes, very desirable because of their ease in cultivar development and increased resistance via combination into a single cultivar, often are quickly lost to pathogen adaptation. However, the *Pi-ta* gene crossed into the 'Katy' cultivar from 'Tetep' (Moldenhauer et al. 1990) provided reliable resistance to the contemporary blast pathogen population for 14 years in Arkansas before being overcome in the new *Pi-ta c*ultivar 'Banks' (Moldenhauer et al. 2007c) by race IE-1k (Lee et al. 2005b). This apparent failure of the *Pi-ta* gene is considered here.

2 Rice Blast in the USA

2.1 Contemporary *M. oryzae* Population

Marchetti (1994) defines a decade-long pathodeme driven pathogen adaptation when characterizing *M. oryzae* pathotypes from southern USA rice production areas. The *M. oryzae*-host relationship is further defined as being eight distinct MGR586 DNA fingerprint lineages with four distinct lineages (A, B, C, and D) predominating in the contemporary population. Multiple haplotypes occur within lineages but typically only one or two haplotypes prevail due to unknown fitness requirements. These investigations defined four distinct vegetative mating types, US-01, US-02, US-03 and US-04, in the contemporary USA blast pathogen population which has an exceptionally low level of genetic diversity relative to *M. oryzae* worldwide (Xia et al. 1993,2000; Correll et al. 2000a,b). Races IB-49 and IC-17 currently predominate in the southern US production fields with races IB-1, IE-1, IG-1, and IH-1 being recovered less frequently. Race IE-1k, rarely detected before 2004, has increased in 'Banks'.

2.2 Partial Blast Resistance

Arkansas breeders utilize partial blast resistance (PBR) in high-yield rice cultivars by retaining and evaluating blast susceptible cultivars. PBR is evaluated using a combination of leaf lesion characteristics (Marchetti 1994) and panicle blast severity in drought stressed upland field nurseries (Bonman 1992; Lee et al. 2006). Following intense performance testing in nurseries and grower field plots, entries with higher yield and desirable agronomic characteristics are released as susceptible cultivars with the understanding that significant yield loss can occur if not properly managed with respect to blast disease.

2.3 Root Zone Induced Field Resistance

High PBR alone does not provide sufficient blast control. Long term PBR efficacy is determined by multiple environmental conditions, particularly those impacting the root zone (Bonman 1992; Lee et al. 2004, 2005a; Singh et al. 2004a,c). A highly effective and cumulative field resistance can be induced in PBR cultivars and mediated by anaerobic conditions (low dissolved oxygen (DO)) established in the root zone during continuous-flood-irrigation. (Lee et al. 2004; Singh et al. 2004a,c). Root zone DO mediates form and quantity of plant nutrients affecting disease susceptibility, production of ethylene and other hormones associated with disease-resistance, and specific morphological changes which enhance oxygen movement to rice roots and inhibits *M. oryzae* growth across vein tissue (Lee et al. 2004; Singh et al. 2004a,b,c). Arkansas rice growers routinely utilize flood-irrigation-induced field resistance as a primary rice blast control strategy (Lee et al. 2006).

3 *M. oryzae* Adaptation to the *Pi-ta* Gene

The *Pi-ta* gene in 'Katy' conferred complete resistance to all contemporary *M. oryzae* pathotypes in the US (Moldenhauer et al. 1990; Marchetti 1994; Correll et al. 2000a; Jia et al. 2004). 'Katy' was planted to 21 percent of Arkansas production in 1992 (Lee 1994). Higher yielding *Pi-ta*-based cultivars including 'Kaybonnet (Gravois et al. 1995), Drew (Moldenhauer et al. 1998) and Ahrent (Moldenhauer et al. 2007d) were planted to a maximum of 35% of total state hectarage during 1998 (Lee et al. 2006). Thereafter, *Pi-ta*-based cultivar planting quickly declined (Wilson and Branson 2003, 2005; Lee et al. 2006) as growers changed to higher-yielding field-resistant cultivars including 'Wells' (Moldenhauer et al. 2007a), 'LaGrue' (Moldenhauer et al. 1994), and 'Francis' (Moldenhauer et al. 2007b).

Virulence adaptation to the *Pi-ta* gene was anticipated and monitored (Marchetti 1994; Correll et al. 2000a,b). All *Pi-ta*-based cultivars released until now are susceptible to laboratory isolates, races IB-33 and IE-1k, in greenhouse tests (Table 1). Collecting only rare random wild isolates, scientists concluded race IE-1k was poorly adapted to field conditions (Correll and Lee 2000, 2000a,b; Xia et al. 2000). Thus, the *Pi-ta* gene provided apparent durable blast resistance to contemporary races until 2004 when a seed production field of the newly released *Pi-ta*-containing cultivar 'Banks' was severely damaged by rice blast (Lee et al. 2005b).

4 Diagnosis of Apparent *Pi-ta* Failure

Research began immediately to determine why the race IE-1k epidemic occurred in 'Banks' during 2004. Investigations centered around a possibilities of a *M. oryzae* virulence adaptation, cultivar predisposal to blast by unusual environmental or cultural conditions, or genetic abnormalities within 'Banks'.

Table 1 Cultivar blast reaction observed in inoculated greenhouse assays to plants growing upland and in non-inoculated drought stressed production fields with race IE-1k present

Cultivar Tested	Resistance genes[a]		Drought stressed upland field rating[b]	Cultivar susceptibility to *M. oryzae* in inoculated greenhouse assays[c]				
	Pi-ta/ Pi-ta[2]	*Pi-kh/ Pi-ks*		Race IB-49	Race IC-17	Race IE-1k	Isolates from Banks	Race IB-33
Katy	*Pi-ta*	*Pi-ks*	R	R	R	S	S	S
Drew	*Pi-ta*	*Pi-ks*	R	R	R	S	S	S
Ahrent	*Pi-ta*	*Pi-ks*	R	R	R	S	S	S
Cybonnet	*Pi-ta*	*Pi-kh*	R	R	R	S	S	S
Banks	*Pi-ta*	NP	VS-S	R	R	S	S	S
Wells	NP	*Pi-kh*	VS-S	S	S	S	S	S
Cypress	NP	*Pi-kh*	S-MS	S	S	S	S	S
Francis	NP	NP	VS	S	S	S	S	S
LaGrue	NP	NP	VS-S	S	S	S	S	S

[a] Resistance gene presence as indicated by SSR marker analysis. NP = not present. 'Francis' is known to contain the *Pi-i* gene. Cultivars test negative for the *Pi-b* and *Pi-z* genes.
[b] Cultivar blast susceptibility rating in moisture stressed production fields with race IE-1k present. VS = very susceptible, S = susceptible, MS = moderately susceptible and R = Resistant.
[c] Cultivar blast susceptibility rating for inoculated greenhouse plants grown under upland conditions. S = susceptible and R = Resistant.

4.1 Virulence Tests

Field isolates collected from diseased 'Banks' plants during 2004 were identified as being race IE-1k in DNA lineage group B, and Vegetative Compatibility Grouping US-02, negative for AVR-Pi-ta gene of the fungus and were compared with known race IE-1k isolates in standard greenhouse bioassays (Lee et al. 2005b). Isolates from 'Banks' samples caused susceptible type leaf lesions on *Pi-ta*-containing cultivars 'Banks', 'Cybonnet' (Gibbons et al. 2006), 'Drew', and 'Katy' and compared with those caused by type race IE-1k (Table 1). Differential cultivars were not found to distinguish between field isolates from 'Banks' and previously known isolates of IE-1k.

4.2 Environment

Field environmental conditions during 2004 were highly conducive for rice blast in seed fields and test plots of 'Banks'. However, severe blast epidemics occurred in 'Banks' production fields during 2005–2007 when environmental conditions were much less conducive for the blast disease. In contrast, blast did not occur in fields of contemporary *Pi-ta*-based cultivars 'Drew', 'Cybonnet' or 'Ahrent' during 2004–2007.

4.3 Cultivar Predisposal and Field Resistance

Drought stress associated with sandy soils and poor flood-irrigation management were identified as primary predisposing conditions for blast in 'Banks'. Samples of conducive soil types were collected for short-term greenhouse tests (Lee et al. 2007). An increased leaf blast severity in drought-stressed plants inoculated with isolate IE-1k occurred with all soil samples but the increase was not specific for soil samples collected from fields where 'Banks' blast epidemics had occurred (Fig. 1). In general, leaf blast was more severe in 'Banks' and compared with that observed in 'Wells'. The flood-irrigation-response for test cultivars growing in the soil samples compared with that of 'LaGrue', and 'Wells' and *Pi-ta*-based 'Katy', 'Kaybonnet', 'Drew' and 'Ahrent' inoculated with race IE-1k (Lee et al. 2005a, 2007) and with that of *Pi-ta*-based cultivars 'Tetep' and 'Tadukan' (Jia et al. 2003) when inoculated with races IE-1k or IB-33 (Lee et al. 2005a).

4.4 Molecular Analysis

Zhou et al. (2007) defined a transposition mutation specific for isolate B2 from 'Banks' as not being clonal to additional *Pi-ta*-virulent wild type isolates from 'Banks' and concluded multiple molecular mechanisms may be operating to defeat the *Pi-ta* gene. The *Pi-ta* allele in Banks was determined to be molecularly identical to the *Pi-ta* allele in 'Katy' (Jia et al. 2007). DNA marker data indicated 'Banks' does not contain the blast resistance *Pi-ks* gene common to 'Katy', 'Drew' and 'Ahrent' or the *Pi-kh* gene contained in 'Cybonnet' (Fjellstrom et al. 2004; R.G. Fjellstrom, personal communication 2008; V.A. Boyett, personal communication 2008).

5 Discussion

Pi-ta-based cultivars provided immunity to rice blast in the USA for 14 years before blast epidemics occurred in 'Banks'. This event initially suggested *M. oryzae* adaptation had defeated the *Pi-ta* gene. However, virulence specific isolates defining a new race shift were not recovered from field samples of 'Banks'. Differential molecular variation observed with 'Banks' field isolate B2 was not associated with recognizable changes in isolate virulence. In the absence of differential cultivars to define a new virulence adaptation and the absence of field epidemics in contemporary *Pi-ta*-based cultivars 'Ahrent', 'Cybonnet' and 'Drew', there is little physical evidence for a *M. oryzae* race shift beyond that originally described over a decade ago for race IE-1k.

Although the Banks *Pi-ta* gene is molecularly identical to that in 'Katy' (Jia et al. 2007), the *Pi-ta* donor is less clear for 'Banks' which was developed through a

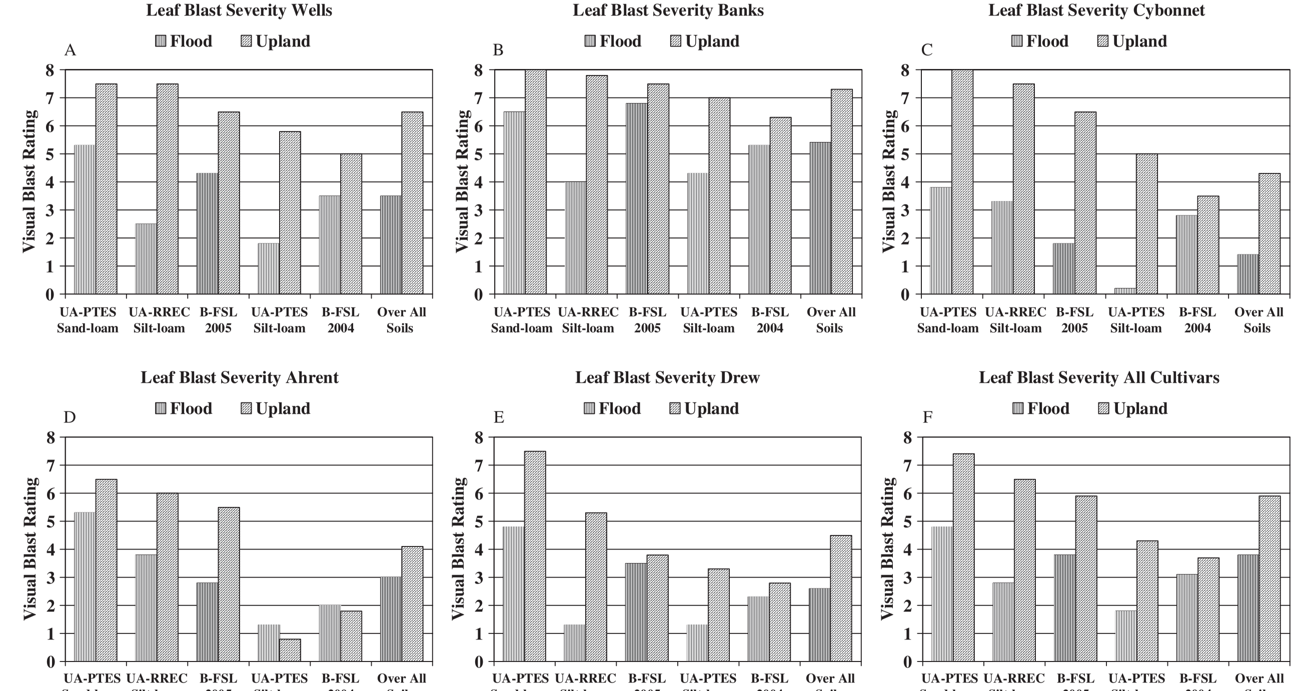

Fig. 1 Leaf blast severity observed in race IE-1k inoculated greenhouse tests to evaluate the impact of soil sample on rice blast severity. Soil samples were: an unknown sandy-loam (UA-PTES Sand-loam) from the University of Arkansas Pine Tree Experiment Station blast nursery (UA-PTES) near Colt, AR, USA; a Dewitt silt-loam (UA-RREC Silt-loam) from the University of Arkansas Rice Research and Extension Center (UA-RREC), Stuttgart Arkansas, USA; a Bosket FSL soil (B-FSL 2005) from a 2005 Banks blast epidemic site; an unknown silt-loam (UA-PTES Silt-loam) from UA-PTES; a Bosket FSL soil (B-FSL 2004) from a 2004 Banks blast epidemic site. Cultivars growing in either upland-drought-stressed or continuous-flood-irrigation cultural treatments were: (**A**) Wells (**B**) Banks, (**C**) Cybonnet, (**D**) Ahrent, (**E**) Drew and (**F**) All cultivar summary

backcross program which primarily retains characteristics of the recurrent parent, in this case 'LaGrue' (Moldenhauer et al. 2007c). The primary selection in the backcross was for race IE-1k blast resistance which has a potential for eliminating lesser resistance genes such as *Pi-ks*, *Pi-kh*, *Ptr (t)* gene and QTL's (K.A.K. Moldenhauer, personal communication 2008; Jia and Martin 2008). Consequently, blast resistance genes *Pi-kh* or *Pi-ks* (Table 1) and possibly other resistance genes are not present in Banks. 'Banks' blast susceptibility in their absence suggests these genes served to sustain the *Pi-ta* gene in field situations by masking the inherent race IE-1k vulnerability in 'Katy', 'Kaybonnet', 'Drew', Ahrent' and 'Cybonnet'.

The specific role of *Pi-ks* or *Pi-kh* genes is unclear. The *Pi-ks* gene in Katy evidently provides resistance to race IB54 (Jia and Martin 2008). The *Pi-kh* gene is identified as a durable blast resistance gene (Sharma et al. 2005). 'Wells', which contains the *Pi-kh* gene but not the *Pi-ta* gene, rates susceptible or very susceptible to race IE-1k and other common blast races in inoculated greenhouse and drought-stressed field tests (Moldenhauer et al. 2007a; Lee et al. 2006). When properly managed to induce cumulative continuous-flood-irrigation resistance, however, 'Wells' exhibits field resistance equivalent to the *Pi-ta* cultivars and has been planted to over 30 percent of Arkansas rice production fields since 2001 (Lee et al. 2006).

The loss of flood-induced-field resistance inciting blast epidemics with drought stressed 'Banks' and the inherent vulnerability in greenhouse tests to race IE-1k strongly suggest that, relative to races IE-1k and IB-33, the *Pi-ta* gene in cultivars 'Katy', 'Ahrent', 'Cybonnet', 'Drew', 'Banks', and 'Tetep' function as a PBR gene which is being complimented by additional minor genes. Regardless, it is important to emphasize the *Pi-ta* gene derived from 'Tetep' as originally deployed in 1990, in conjunction with minor resistance genes, has served as a valuable source of practical immunity against the rice blast disease without being compromised during highly conducive field environments.

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Resistance Genes and Their Effects to Blast in Korean Rice Varieties (*Oryza sativa* L.)

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Abstract A total of 98 varieties consisting of 88 *japonica* and 10 *Tongil-type* of rice were analyzed to resistant genes and their effects to blast. The 13 major blast resistance (*R*) genes against *Magnaporthe oryzae* were screened in a number of Korean rice varieties using molecular markers. Twenty-eight (28.6%) were found to contain the *Pia* gene originating from Japanese *japonica* rice genotypes. The *Pib* gene from BL1 and BL7 was incorporated into 39 Korean *japonica* varieties, whereas this same gene from the IRRI-bred *indica* varieties was detected in all *Tongil-type* variety. The *Pii* gene was found in 17 of the *japonica* varieties. The *Pii* gene in Korean rice varieties originates from the Korean *japonica* variety Nong-baeg, and Japanese *japonica* varieties Hitomebore, Inabawase, and Todorokiwase. The *Pi5* gene, which clusters with *Pii* on chromosome 9, was identified only in an *Tongil-type*, Taebaeg. Thirty-four varieties were found to contain alleles of the resistance gene *Pita* or *Pia-2*. The *Pita* gene in *japonica* varieties was inherited from the Japanese *japonica* genotype Shimokita, and the *Pita-2* gene was from Fuji280 and Sadominori. Seventeen *japonica* and one *Tongil-type* varieties contained the *Piz* gene, which in the *japonica* varieties originates from Fukuhikari and 54BC-68. The *Piz-t* gene contained in three *Tongil-type* varieties was derived from IRRI-bred *indica* rice varieties. The *Pi9(t)* gene locus that is present in Korean *japonica* and *Tongil-type* varieties was not inherited from the original *Pi9* gene from wild rice *Oryza minuta*. The *Pik*-multiple allele genes *Pik*, *Pik-m*, and *Pik-p* were identified in 24 of the varieties tested. The *Pit* gene inherited from the *indica* rice K59 was not found in any of the Korean rice varieties tested. In haplotype analysis for the loci related to two QTLs as well as five major resistant genes and, two *R* genes *Piz* and *Pita* showed stable resistant effects to blast nurseries and isolates in Korea.

Keywords Resistance gene · Blast · Rice variety · *Japonica* · *Tongil-type* · Haplotype

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1 Introduction

Rice is a staple food in Korea and continuous research efforts have therefore been made to produce varieties with improved levels of yield potential and the resistance to diseases and insects. Since 1960 in Korea, 46 *Tongil-type* varieties, that are derived from the crosses between *indica*- and *japonica-type* subspecies, and a further 160 *japonica* varieties have been developed. In the early 1970s, *Tongil-type* varieties were released for commercial use, and occupied more than 75% of the total rice cultivation areas by the end of 1970s. The proliferation of diverse genotypic varieties of rice with enhanced disease resistance, however, has triggered genetic mutations leading to the differentiation of the blast fungus *Magnaporthe grisea*. This in turn has led to break down and loss of blast resistance in *Tongil-type* rice varieties in 1977. Several commercial varieties of *japonica* rice were also developed in the mid 1980s and were adopted for cultivation due to needs of consumer and farmer on the high yield and good eating-quality. As a result, little cultivated areas of the *Tongil-type* varieties have been recorded in Korea since 1990. Significantly, the same virulent blast isolates also caused great damage to *japonica* varieties in 1985 and 1993, and especially in 1999, three *japonica* rice varieties including Daesan, Dongan, and Ilmi, were found to lose their resistance to leaf and neck blast isolates (Han et al. 2001). Ultimately, we experienced the breakdown of the resistance to blast in about 7- to 10-year cycles since 1978 with the rapid increase of cultivation areas of varieties of similar genetic background such as *Tongil-type* or *japonica* varieties by the differentiation of new isolates of the enhanced virulence.

Little is currently known about blast resistance genes contained among Korean rice varieties as only a few strains have so far been analyzed for the presence of these genes (Ahn et al. 1997; Kwon et al. 2002; Cho et al. 2004; Yi et al. 2004) or their haplotypes (Hwang et al. 2004). Most of the developed Korean *japonica* varieties have been found to be derived from Japanese *japonica* parents that contain the blast resistance genes, *Pia*, *Pib*, *Pii*, *Pik* multiple alleles, and *Pita* based on the pedigree of the breeding lines (Choi et al. 1989). Our current study was undertaken to expand upon the existing knowledge in this area by identifying the major resistance (*R*) genes to *M. grisea* from Korean *japonica* and *Tongil-type* varieties and to evaluate their effects in the field.

2 The Major Resistance Genes to *M. grisea* in Korean Rice Varieties

Among the 98 varieties analyzed, 87 (88.8%) were found to have at least one of the 13 major *R* genes analyzed (Table 1). The four *R* genes *Pia*, *Pii*, *Pik*, and *Pik-m* of 13 resistance genes analyzed originated from *japonica* rices, and identified only from *japonica* varieties in our current analyses. Three genes *Pia, Pii*, and *Pik* were originally inherited from the Japanese *japonica* varieties Aichi Asahi, Fujisaka5, and Kanto51, respectively (Yamasaki & Kiyosawa 1966), and *Pik-m* gene originated

Table 1 *M. grisea* resistance genes and their donors based on molecular marker analysis of 98 Korean rice varieties

R genes	Gene donors	Major varieties[a]	No. of varieties (%)
Pia	Aichi37, Asominori, Fuji269, Fuji280, Jinheung, Kanto100, Kimmaze, Kuiku90	Dongjin, Mangeum, Moonjang, Palgong, Saesangju, Samgwang, Seomjin, Sinseonchal	29 (29.6)
Pib	BL1, BL7, IR8, IR24, IR29	Daepyeong, Daesan, Dongan, Dongjin1, Geuman, Gopum, Hwayeong, Ilpum, Junam, Palgong, Samgwang, Sangju, Sangmi, Seomjin, Sindongjin, *Gaya, Milyang23, Anda, Dasan*	49 (50)
Pii	Nongbaeg, Hitomebore, Inabawase, Todorokiwase	Gopum, Hopyeong, Ilpum, Jinpum, Manchu, Nampyeong, Seoan, Sobi, Taebong	17 (17.3)
Pi5		*Taebaeg*	1(1.0)
Pita, *Pita-2*	Shimokita (*Pita*)	Sambaeg, Gopum, Nampyeong, Seojin, Sampyeong, Sangmi, Dongjinchal, Sangjuchal, *Gaya*	34 (34.7)
	Fuji280 (*Pita-2*), Sadominori (*Pita-2*)	Gru, Moonjang, Saesangju, Dongjin, Gyehwa, Ilmi, Jungsan	
Piz	Fukuhikari, 54BC-68	Gopum, Hwanam, Moonjang, Saesangju, Taeseong, *Gaya*	18 (18.4)
Piz-t	IR2061, IR4445	*Baegunchal, Hangangchal, Samgang*	3 (3.1)
Pi9(t)[b]		*Anda*, Undoo, Gru, Moonjang, Sangju, Saesangju, Sambaeg, Sangmi, Goun, Jinbu, Unbong, Geumo	21 (21.4)
Pik	Hokuriku109, Akitsuho	Odae, Jinbu, Tamjin, Gru, Jungsan, Gopum, Goun, Moonjang, Junghwa, Seogan	15 (15.3)
Pik-m		Seogan	1 (1.0)
Pik-p		Sangnambat, *Tongil, Milyang23, Taebaeg*	8 (8.2)
Pit			0 (0)

[a] The varieties listed in italics are *Tongil-type* developed from the cross between *indica* and *japonica*.
[b] The *Pi9(t)* locus is distinct from the original *Pi9* resistance locus.

from the Japanese *japonica* variety Tsuyuake (Kiyosawa 1978). The *Pia* gene on chromosome 11 was identified in 29 *japonica* rice varieties. The donor of this gene was found principally to be the seven Japanese *japonica* rice genotypes, Aichi37, Asominori, Fuji269, Fuji280, Kanto100, Kimmaze, and Kuiku90, and a Korean *japonica* variety Jinheung. This gene was not identified among the 10 *Tongil-type* varieties. The 17 *japonica* varieties were classified as containing the *Pii* gene. The primary donors of the *Pii* resistance gene were found to be Nongbaeg from a Korean *japonica* variety, and Hitomebore, Inabawase, and Todorokiwase from Japanese *japonica* varieties. The major *japonica* cultivars harboring the *Pii* gene were found to be Gopum, Hopyeong, Ilpum, Sobi, and Taebong. Out of the *Pik*-multiple alleles,

Pik, *Pik-p*, and *Pik-m*, the *Pik-p* gene was identified in a *japonica* variety Sangnambat, and seven *Tongil-type* varieties. The *Pik* gene was identified in 17 *japonica* varieties but not in *Tongil-type* varieties, and the *Pik-m* gene was detected only in a *japonica* Seogan. However, we were not able to deduce the donor parents for these *Pik-m* and *Pik-p* genes in *japonica* varieties without the use of an allelism test.

The other eight genes, *Pib*, *Pi5*, *Pita*, *Pita-2*, *Piz*, *Piz-t*, *Pik-p*, and *Pit* in this study originated from *indica* rices, and an *R* gene *Pi9* was from wild rice *Oryzae minuta*. The *Pib* gene on chromosome 2 is an *indica*-derived resistance gene, and introgressed independently from two Indonesian and two Malaysian varieties (Yokoo et al. 1978). The donor isogenic lines for *Pib* gene in Korean *japonica* rice varieties were BL1 from an Indonesian *indica* variety Tjahaja, and BL7 derived from the Malaysian *indica* Milek Kuning strain, respectively. We further found that 49 of the varieties tested in this study harbored the *Pib* gene on chromosome 2. The donors of this gene among the *japonica* group included two Japanese isogenic lines, BL1 and BL7. In contrast, the *Pib* gene detected in the *Tongil-type* varieties was found to originate from the IRRI-bred *indica* varieties IR8, IR24, and IR29. It should be noted that all of the *Tongil-type* cultivars examined in our current study harbor the *Pib* gene. Two *R* genes *Pita* and *Pita-2* on chromosome 12 were introgressed from a common donor, the *indica* Philippine variety Tadukan into various Japanese *japonica* rices (Shigemura & Kitamura 1954). *Pita* was mapped to the position overlapping *Pita-2* by graphical genotype analysis of an NIL with a very narrow introgressed region, Shimokita (Rybka et al. 1997). This result was consistent in that these functionally related genes are allelic or at least very closely located, and may be derived from a common ancestral gene. Thirty four (34.7%) of the 98 rice varieties was found to harbor *Pita* and *Pita-2* gene. The primary donor of the *Pita* gene among the Korean *japonica* rice varieties was found to be from Shimokita, and *Pita-2* gene was from Fuji280 and Sadominori. Interestingly, the three varieties Dongjinchal, Gopum, and Suweon480 produced positive bands with all of the primer sets specific for the resistant *Pita*-alleles and the susceptible *pita*-allele. The *Pita* gene-alleles in Gopum and Suweon480 were introduced from Shimokita and Fuji280, respectively, but the origin of this gene in Dongjinchal could not be inferred from the parent lines. Of the 10 *Tongil-type* varieties screened, only Gaya was positive for the *Pita*-allele markers. The *Piz* gene derived from the USA *indica* rice Zenith was introduced into the Japanese *japonica* rice varieties Fukuhikari and 54BC-68 (Kiyosawa 1967), and this gene in Korean *japonica* varieties was from Fukuhikari and 54BC-68. The *Piz-t* gene in the IRRI-bred *indica* lines originated from the Indian *indica* varieties, TKM1 and TKM6 (Ebron et al. 2004), and this gene in *Tongil-type* was derived from TKM6 based on pedigree tracking. The *Piz* and *Piz-t* genes on chromosome 6 were identified from the 21 Korean varieties in our study cohort. The *Piz* gene using these Japanese *japonica* varieties as primary donors was introduced into Korean *japonica* varieties. Three Korean *Tongil-type* varieties, Baegunchal, Hangangchal and Samgang, contained *Piz-t* gene inherited from the IRRI-bred *indica* lines, IR2061 and IR4445. The *Pik-p* gene which was first reported in the west Pakistani *indica* rice variety Pusur (Kiyosawa 1969), could be inherited into seven *Tongil-type* varieties from the IRRI-bred *indica* lines. The *Pik-p*

gene identified in seven *Tongil-type* varieties could be inherited from the IRRI-bred *indica* lines. The resistance gene *Pit* on chromosome 1 was inherited from an *indica* rice strain K59 (Kiyosawa 1972). None of the Korean rice varieties analyzed in the present study were found to be positive for the SNP marker of the *Pit* gene, t256, which is inherited from an *indica* rice strain K59. The *Pi5* gene was identified from a cross between CO39 and Moroberekan (Wang et al. 1994). In this study, this gene was only identified in *Tongil-type* variety Taebaeg. Out of 98 varieties tested, the *Tongil-type* variety Taebaeg was produced a positive band for the *Pi5*-specific dominant marker JJ817 (Cho et al. 2007). In this result, we speculate that the *Pi5* gene in the Taebaeg (*Tongil-type*) variety might have been inherited from IRRI-bred *indica* rice.

The *Pi9* gene locus was discovered in *Oryza minuta*, a tetraploid wild species of the *Oryza* genus (Amante-Bordeos et al. 1992). The Korean *japonica* and *Tongil-type* varieties were grouped into four types Koshihikari-type, *Piz-t* and *Piz-5*-type, *Piz* and *Pi9*-type, and null-type, respectively, from two markers, pBA14 and NBS2-O/U (Fig. 1). The 21 varieties of *Piz* and *Pi9*-type were not positive to the 195-1 marker, but the monogenic line IRBL9-W of *Pi9* gene was positive. As a result, the *Pi9(t)* gene locus from the Korean *japonica* and *Tongil-type* varieties differs from the original *Pi9* gene, indicating that it may be a member of a multi *R* gene family. Twelve out of the 21 varieties tested that contain the *Pi9(t)* gene cluster were found to contain the *Piz* gene. The loci of this cluster are located on chromosome 6 and form a region containing the *Pi9*, *Piz*, and *Piz-t* genes, of which the *Pi9* and *Piz-t* genes are closely related in sequence and structure to the multiple gene family members at their corresponding loci (Qu et al. 2006; Zhou et al. 2006). The 21 Korean varieties of *Piz* and *Pi9*-type were not positive to 195-1 marker, but the monogenic line IRBL9-W of *Pi9* gene was positive. We designated this locus as

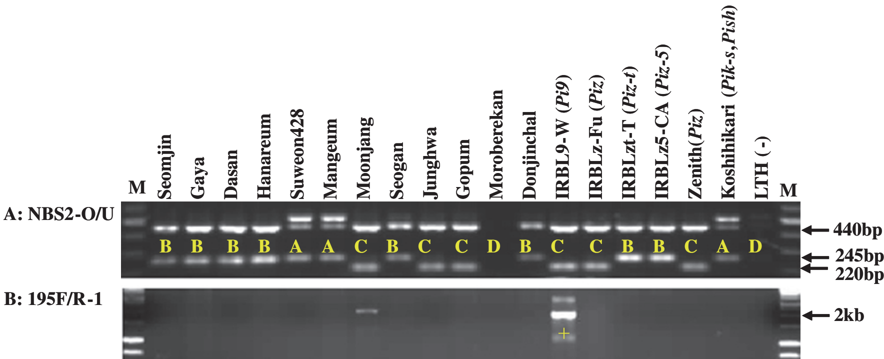

Fig. 1 1 PCR profiles from genomic DNA amplified by two dominant primers for NSB2-Pi9 (Qu et al. 2006). (**A**) The PCR products that produced using NBS2-O/U primer set were digested by the restriction enzyme Hinf I and classified into four types, type A of 500-460-240 bp bands, type B of 460-240 bp bands, type C of 460-220 bp bands, and type D of null band, respectively. (**B**) 195F-1/R-1 primer for NSB2-Pi9 candidate gene produced the positive band for IRBL9-W

Pi9(t) in 21 Korean rice varieties because it might not be *Pi9* gene but possibly a member of a multigene family of resistance loci.

3 Classification of Rice Varieties Based on Nursery Screening for Blast Resistance

The reactions to *M. grisea* among the 88 *japonica* and 10 *Tongil-type* varieties under study were classified into five categories based on four-year nursery test undertaken in 14 local experimental plots in Korea from 2003 to 2006: (a) resistant (R); (b) resistant moderately (RM); (c) medium resistant (M); (d) moderately susceptible (MS); (e) susceptible (S). The incidence of blast disease was scored from 0 (no lesions) to 9 (necrosis of all leaves and sheaths) using IRRI standard evaluation method. Varieties with scores of 0–3 in over 70% of the plots analyzed were assigned to the resistance (R) group, and those with scores of 0–3 in 50–70% of the plots and of 4–6 in the rest plots were placed in a resistant moderately (RM) group. Varieties with scores of 0–3 or 4–6 in over 80% plots were assigned to moderately resistance (M). Varieties with scores of 7–9 in less 40% plots and of 0–3 and/or 4–6 scores in the rest plots were indicated in a moderately susceptible (MS), and those with scores of 7–9 in over 40% of the plots indicated that the strains belonged to the susceptible (S) group.

Among the 88 *japonica* varieties tested, 23 varieties were classified as resistant and resistant moderately, 33 as moderately resistant, and 21 as moderately susceptible and susceptible varieties (Tables 2–4). The 23 varieties of resistant group consisted of 12 early maturing, 8 medium, and 3 mid-late varieties, while the varieties of medium resistance included 9 early, 4 medium and 20 mid-late maturing, and the susceptible varieties were 5 early, 9 medium and 7 mid-late maturing. The remaining 11 *japonica* varieties were not classified into any of these three groups since they do not contain any of the 13 major blast resistant genes (Table 5). Ten *Tongil-type* varieties were classified as resistant and resistant moderately (R and RM) at the blast nursery screening (Table 6). Three NPTs of *Tongil-type* showed resistant moderately (RM) with scores of 0–3 in most of the plots or of 4–5 in a few plots.

3.1 R Genes Associated with the Resistant Group

The results obtained with the resistant group consisting of 23 *japonica* rice varieties (Table 2) suggested that 19 of these varieties had two to four *R* genes. Two varieties, Donghae and Sinseonchal contained one *R* gene *Pia* and *Pii* respectively. Taebong of early maturing harbored five *R* genes, *Pib*, *Pii*, *Piz*, *Pik*, and *Pi9(t)*, while Gopum variety of good palatability and medium maturity had five *R* genes, *Pib*, *Pita*, *Piz*, *Pik*, and *Pi9(t)*. Four early-maturing varieties had four *R* genes each; Gru (*Pib*, *Pita*, *Pik*, *Pi9(t)*), Moonjang (*Pia*, *Pita*, *Pik*, *Pi9(t)*), Saesangju (*Pia*, *Pita*, *Piz*, *Pi9(t)*),

Table 2 Major *R* genes present in *japonica* rice varieties of resistance at blast nursery test

Varieties	Ecotype[a]	Line no.	*R* genes	Reaction
Gru	*E*	Suweon416	*Pib, Pita-2, Pi k, Pi9(t)*	R
Moonjang	*E*	Sangju21	*Pia, Pita-2, Pik, Pi9(t)*	R
Saesangju	*E*	Sangju24	*Pia, Pita-2, Piz, Pi9(t)*	R
Samcheon	*E*	Unbong13	*Pia, Pi z, Pi9(t)*	R
Sangmi	*E*	Sangju19	*Pib, Pita, Pi9(t)*	R
Undoo	*E*	Jinbu25	*Pib, Pi z, Pi9(t)*	RM
Jinbu	*E*	Jinbu10	*Piz, Pita, Pik, Pi9(t)*	RM
Manchu	*E*	Iksan448	*Pita-2, Pii, Pik*	RM
Sambaeg	*E*	Sangju12	*Pia, Pita, Piz, Pi9(t)*	RM
Suweon365	*E*	Suweon365	*Pia, Pib, Pita-2*	RM
Taebong	*E*	Cheolweon59	*Pib, Pii, Piz, Pik, Pi9(t)*	RM
Taeseong	*E*	Cheolweon61	*Piz, Pik, Pi9(t)*	RM
Donghae	*M*	Yeongdeog5	*Pia*	RM
Geuman	*M*	Suweon462	*Pib, Pita-2*	RM
Gopum	*M*	Suweon479	*Pib, Pita, Piz, Pii, Pik*	RM
Manweol	*M*	Milyang173	*Pia, Pib*	RM
Palgong	*M*	Milyang80	*Pia, Pib*	RM
Sampyeong	*M*	Suweon444	*Pib, Pita*	RM
Sangnambat	*M*	Milyang93	*Pib, Pik-p, Pi9(t)*	RM
Sangok	*M*	Milyang182	*Pia, Pita*	RM
Seogan	*L*	Namyang6	*Pita-2, Pik, Pik-m*	RM
Sinseonchal	*L*	Iri355	*Pia*	RM
Sujin	*L*	Milyang156	*Pib, ta-2*	RM

[a] Ecotype: *E*, early maturing; *M*, medium maturing; *L*; mid-late maturing.

Jinbu (*Piz*, *Pita*, *Pik*, and *Pi9(t)*), and Sambaeg (*Pia*, *Pita*, *Piz*, *Pi9(t)*). Among the 23 *japonica* varieties belonging to the resistant group, 12 (52.2%) harbored the *Pib* gene, 14 (60.1%) contained the *Pita* or *Pita-2* genes. The *Pia*, *Pii*, *Piz*, *Pik*, *Pik-m*, and *Pik-p* genes were identified from 10, 3, 8, 8, 1, and 1 of these varieties, respectively. The *Pi9(t)* gene, a putative member of the multiple *R* gene family, was identified in 11 (47.8%) out of 23 resistant *japonica* varieties.

3.2 R Genes Contained in the Moderately Resistant Group

Thirty-three *japonica* varieties were assigned to the moderately resistant group at blast nursery test (Table 3). The resistance genes in this group were *Pia* in 10 varieties, *Pib* in 20, *Piz* in 7, *Pii* in 7, *Pik* in 5, and *Pita* or *Pia-2* in 12 further varieties. Sixteen varieties contained a single *R* gene, whereas 13 varieties contained two to three resistance genes. Two varieties, Joan and Sangjuchal contained five *R* genes, and two other varieties, Junghwa and Sangju harbored four *R* genes. Among the varieties containing a single *R* gene, *Pia* was found in two, *Pib* in nine, *Pita* and *Pii* in two of these strains, and *Piz* in one variety. A putative *R* gene of the multigene family of the *Pi9(t)* locus was identified in seven of the *japonica* varieties in this moderately blast resistant group. Two varieties, Palgong and Seomjin were

Table 3 Major *R* genes present in *japonica* rice varieties of moderate resistance at blast nursery test

Varieties	Ecotype[a]	Line no.	*R* genes	Reaction
Goun	*E*	Jinbu36	*Piz, Pik, Pi9(t)*	M
Jinbuchal	*E*	Jinbu9	*Piz, Pii*	M
Joan	*E*	Suweon478	*Pia, Pib, Pii, Pik, Pi9(t)*	M
Junghwa	*E*	Sangju15	*Pib, Pita-2, Pik, Pi9(t)*	M
Sangju	*E*	Sangju10	*Pia, Pib, Piz, Pi9(t)*	M
Sangjuchal	*E*	Sangju18	*Pita, Piz, Pii, Pik, Pi9(t)*	M
Sinunbong	*E*	Unbong7	*Piz*	M
Suweon345	*E*	Suweon345	*Pib*	M
Unbong	*E*	Unbong1	*Pia, Piz, Pi9(t)*	M
Haepyeong	*M*	Yeongdeog26	*Pib, Pi9(t)*	M
Seoan	*M*	Namyang6	*Pib, Pii*	M
Sobi	*M*	Iksan435	*Pii*	M
Yeonghae	*M*	Yeongdeog19	*Pib*	M
Daepyeong	*L*	Iksan450	*Pib*	M
Daesan	*L*	Milyang142	*Pia, Pib, Pita*	M
Dongan	*L*	Iksan418	*Pia, Pib, Pita-2*	M
Dongjin	*L*	Iri348	*Pia, Pita-2*	M
Dongjin 1	*L*	Iksan444	*Pib*	M
Gyehwa	*L*	Gyehwa3	*Pia, Pita-2*	M
Hojin	*L*	Iksan436	*Pib, Pita-2*	M
Hwajung	*L*	Suweon387	*Pia*	M
Hwanam	*L*	Milyang115	*Pita*	M
Hwasin	*L*	Iri407	*Pii*	M
Hwayeong	*L*	Milyang101	*Pib*	M
Ilmi	*L*	Milyang122	*Pita-2*	M
Junam	*L*	Milyang165	*Pib*	M
Nampyeong	*L*	Iri416	*Pib, Pita, Pii*	M
Saegyehwa	*L*	Gyehwa19	*Pib*	M
Samgwang	*L*	Suweon474	*Pia, Pib*	M
Seomjin	*L*	Iri353	*Pia, Pib, Pita, Piz*	M
Seopyeong	*L*	Gyehwa22	*Pib*	M
Sindongjin	*L*	Iksan438	*Pib*	M
Tamjin	*L*	Iri373	*Pita, Pik*	M

[a] Ecotype: *E*, early maturing; *M*, medium maturing; *L*; mid-late maturing.

evaluated as the durable resistance varieties from the result of long-term blast nursery screening and sequential planting method (Han et al. 2001; Kim et al. 2004). Palgong was found to have two *R* genes, *Pia* and *Pib*, whereas Seomjin harbored four *R* genes *Pia*, *Pib*, *Pita*, and *Piz*.

3.3 R Genes Present in the Blast Susceptible Group

Among the 88 *japonica* varieties of rice that we analyzed in this study, 21 were assigned to the blast susceptible group based on their nursery screening score (Table 4). Among the resistance genes that were contained in the varieties of this

Table 4 Major *R* genes present in *japonica* rice varieties of different degrees of susceptibility at blast nursery test

Varieties	Ecotype[a]	Line no.	*R* genes	Reaction
Geumo	*E*	Suweon313	*Piz, Pi9(t)*	MS
Hwadong	*E*	Suweon409	*Pia, Pib, Pik*	MS
Jinmi	*E*	Suweon349	*Pib, Pii*	S
Jungsan	*E*	Sangju22	*Pib, Pita-2, Piz, Pik*	MS
Odae	*E*	Suweon303	*Pik*	MS
Gwangan	*M*	Suweon429	*Pia, Pii*	MS
Hwaan	*M*	Suweon447	*Pita*	MS
Hwabong	*M*	Milyang138	*Pia, Pita*	MS
Hwajin	*M*	Suweon346	*Pia*	MS
Hwaseong	*M*	Suweon330	*Pia*	S
Jinpum	*M*	Suweon434	*Pib, Pii*	MS
Jungan	*M*	Suweon438	*Pik, Pi9(t)*	S
Seogjeong	*M*	Namyang26	*Pia*	S
Seojin	*M*	Namyang17	*Pita*	S
Dongjinchal	*L*	Iksan425	*Pita*	S
Hopyeong	*L*	Iri401	*Pii*	S
Hwamyeong	*L*	Suweon423	*Pia*	MS
Ilpum	*L*	Suweon355	*Pib, Pii*	S
Jongnam	*L*	Milyang169	*Pia, Pita-2*	MS
Mangeum	*L*	Iri390	*Pia, Pib, Pii*	MS
Suweon480	*L*	Suweon480	*Pib, Pii*	S

[a] Ecotype: *E*, early maturing; *M*, medium maturing; *L*; mid-late maturing.

Table 5 *Japonica* rice varieties containing none of 13 *R* genes under study

Varieties	Ecotype*	Line no.	*R* genes	Reaction
Namil	E	Suweon472	–	MS
Geumo 1	M	Milyang125	–	M
Hwaseonchal	M	Suweon384	–	MS
Juan	M	Suweon383	–	S
Samdeog	M	Yeongdeog32	–	M
Sura	M	Suweon427	–	MS
Anjung	L	Suweon362	–	M
Daean	L	Suweon396	–	M
Hwasam	L	Milyang123	–	M
Nagdong	L	Milyang15	–	MS
Milyang95	L	Milyang95	–	MS

* Ecotype: *E*, early maturing; *M*, medium maturing; *L*; mid-late maturing.

group, *Pia* was found in 9 varieties, *Pib* in 7, *Piz* in 2, *Pii* in 7, *Pik* in 4, and *Pita* or *Pita-2* in 6 varieties. Furthermore, 19 of 21 varieties in this group contained either a single or two *R* genes. A Mangeum was found to contain three *R* genes, *Pia*, *Pib*, and *Pii* with one further variety Jungsan harboring four *R* genes, *Pib*, *Pik*, *Pita*, and *Piz*. Two varieties, Geumo and Jungan contained a locus corresponding to the *Pi9*-multigene family.

Table 6 Major *R* genes present in Korean *Tongil-type* rice varieties under study

Varieties	Ecotype[a]	Line no.	R genes	Reaction
Baegunchal	*E*	Iri344	*Pib, Piz-t, Pik-p*	RM
Taebaeg	*E*	Suweon287	*Pib, Pi5, Pik-p*	R
Gaya	*M*	Milyang54	*Pib, Pita, Piz*	R
Hangangchal	*M*	Suweon290	*Pib, Piz-t, Pik-p*	R
Samgang	*M*	Milyang46	*Pib, Piz-t, Pik-p*	R
Tongil	*M*	Suweon213	*Pib, Pik-p*	R
Anda[b]	*M*	Suweon431	*Pib, Pik-p, Pi9(t)*	RM
Dasan	*M*	Suweon405	*Pib*	RM
Hanareum	*M*	Milyang181	*Pib*	RM
Milyang23	*L*	Milyang23	*Pib, Pita, Pik-p*	RM

[a] Ecotype: *M*, medium maturing; *M*, medium maturing; *L*; mid-late maturing
[b] Varieties of italic were developed from the crosses between *Tongil-type* and New Plant Type (NPT).

3.4 Non-R Gene Containing Group

Eleven (11.2%) of the *japonica* varieties analyzed appeared not to contain any of the 13 *R* genes screened in this study (Table 5). These varieties were consisted of one early maturing, five medium, and five mid-late. Five varieties were grouped to moderately resistance, another five varieties were moderately susceptible, and the last variety Juan was grouped to susceptible. Nagdong, a highly susceptible Korean *japonica* variety compatible to most Korean blast isolates, was grouped to moderately susceptible at blast nursery test, and not positive for any of the *R* gene-specific markers, as it did not contain any *R* gene source from its parents, Norin6 and Mineyudaka. Although these varieties did not contain any of the blast resistance genes tested, the possibility that they harbored other as yet unknown resistance gene(s) could not be discounted. Other varieties of this group were developed by using most parents having any specific *R* genes, but we suggested that the *R* genes flowed out through the breeding.

3.5 R Genes Present in Tongil-Type (Indica/Japonica) Varieties

All 10 *Tongil-type* rice varieties in this study harbored 1 to 3 *R* genes. *Pib* gene was identified in all *Tongil-type* varieties (Table 6). Three *R* genes, *Pik-p*, *Pita*, and *Piz-t* genes inherited from the *indica* rice genotypes were identified from 7, 2, and 3 of these varieties, respectively. The *R* gene *Piz* was identified only in Gaya. Two varieties, Dasan and Hanareum, of three new high-yielding *Tongil-types* developed from the crosses with IRRI-bred NPT types (New Plant type) were found to contain the *Pib* gene and the other, Anda, contained three *R* genes, *Pib*, *Pik-p*, and *Pi9(t)*. The *Pi5* gene was identified only in Taebaeg.

4 The Effect of *R* Genes Based on Haplotype Analysis

The haplotypes for 56 Korean *japonica* varieties, two *Tongil-type* (*indica*/*japonica*), and two foreign rices Zenith and Moroberekan, were analyzed based on the significant analysis between the resistance reaction by phenotyping and the allele type of DNA markers located at the regions of resistant genes/QTLs to blast (Table 7). The loci of two genes, *Pita* and *Piz*, were significant as stable resistant effect across regions and isolates. The *Pita* gene was significant to five regions and two isolates except for an isolate 97–227, and explained 13.2 ∼ 48.5% of phenotypic variations. The alleles of *Piz* gene were significant to four regions except for Suwon, and three isolates, and explained 12.5 ∼ 40.5% of phenotypic variations. The alleles of a QTL *qBL4.2* and two genes *Pia* and *Pib* were significant to 3 ∼ 4 regions or isolates.

Table 7 ANOVA analysis of DNA markers located at the chromosome regions of two QTLs and five major genes of resistance to rice blast

			Blast nursery test								Isolates					
Gene/ QTLs			Jecheon		Cheolwon		Suwon		Namyang		03-177		97-227		90-008	
	Markers	Chr.	Sig.	$R^{2\#}$	Sig.	R^2	Sig.	R^2	Sig.	R^2	Sig.	R^2	Sig.	R^2	Sig.	R^2
qBL1	RM302	1														
	RM297												*	21.8		
	RM265															
	RM315															
qBL4.2	RM3217	4														
	R746		*	19.2									*	16.5		
	S20518		***	20.4	*	10.4										
	RM255															
	RM131		*	14.1												
	RM1272															
Pia	RM5704	11					*	25.3							*	26.1
	RM120															
	RM6894						**	23.6								
	RM441		*	17.8												
Pib	RM208	2			*	18.2							***	31.8		
	RM406															
	RM482															
	RM207						*	19.2					**	32.6		
	RM48		***	43.7			**	25.9								
	RM138		**	11.0			*	11.7					*	9.9		
Pii	RM296	9														
(*Pi5*)	RM105															
	RM524															
	RM460															
Pita	RM101	12	**	15.8	**	31.9	***	48.5	**	35.1	**	33.1			****	42.4
(*Pita-2*)	OSR32				***	27.5	*	10.7	***	39.8	***	25.2			***	34.6
	RM155				*	12.9	*	13.2	*							
	RM1337						*	16.2							**	27.9
Piz	RM527	6	**	26.8	***	23.5				22.4	****	39.4	*	12.5	****	26.4
(*Pi9*)	RM5850		*	25.8	***	27.9			***	24.4	***	33.5			**	22.5
	RM541				*	22.4			**		*	23.1	****	40.5	*	18.9

$Sig: *, **, *** and **** mean significant at 5%, 1%, 0.1% and 0.01%, respectively.
#R^2(%): percentage explained for a total of phenotypic variation.

A QTL *qBL1* was significant only to an isolate 97–227, however, the *Pii* gene was not significant to any regions and isolates.

5 Conclusion

In 77 *japonica* varieties having over at least one *R* gene, 26 early maturing varieties had an average of 3.2 *R* genes in each, 21 medium maturing had 1.8, and 30 mid-late maturing contained 1.7, respectively. The resistance varieties to blast was 46.2% in early maturing, 38.1% in medium maturing, and 10% in mid-late maturing varieties, respectively. In three ecotypes, most early-maturing varieties harbored more *R* genes than the medium and mid-late maturing ones, and showed a medium to high resistance. Fifteen out of the 17 *japonica* varieties that were found to harbor the *Piz* gene in our present study are early maturing varieties that have been adapted to mid-mountainous or mountainous areas. Only two *japonica* varieties, Gopum and Seomjin which contain *Piz* gene are classified as medium and mid-late maturing, respectively. Also, eighteen out of 21 *japonica* varieties harboring the *Pi9(t)* gene were early-maturing, and three were medium-maturing ones. The genes *Piz*, *Piz-t* and *Pi9* were identified to link closely on chromosome 6 (Qu et al. 2006; Jeung et al. 2007). These results were related that the *Piz* gene has been linked to the major photoperiod sensitivity gene *Se1*, which is responsible for late heading and located on chromosome 6 (Yokoo 2005). Interestingly, most varieties having *Piz* and/or *Pi9(t)* genes showed more stable resistance (Tables 2–4). Also, based on these results, although the phenotypes did not always appear to depend on the number of *R* genes present in a particular variety, many rice varieties having more *R* genes showed higher and stable resistance.

In the screening of 26 monogenic lines of *R* genes to blast (Tsunematsu et al. 2000), the genes originating from *japonica* genotypes were not effective to the blast isolates, and a few genes, *Piz-5*, *Piz-t*, *Pi5*, and *Pi9*, derived from *indica* and wild rice showed stable resistance in Korea (Cho et al. 2005). In haplotype analysis, two genes *Piz* and *Pita* showed stable resistant effects to blast nurseries and isolates. Based on these results, we suggest that the pyramiding of multiple loci of *Piz/Piz-t* and *Pi9* on chromosome 6, *Pi5* on chromosome 9 and *Pita/Pita-2* on chromosome 12 would be the most effective for developing stable and broad-spectrum resistant rice variety in Korea.

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Genetic Analysis of Resistance Against Bacterial Leaf Blight and Leaf Blast Disease in the Japanese Rice Cultivar Asominori

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Abstract The Japanese rice cultivar Asominori shows high bacterial leaf blight resistance (BLBR). The resistance locus is closely linked with the *Ph* gene on chromosome 4. Japanese rice breeders noticed that lines resistant to both bacterial leaf blight (BLB) and rice leaf blast (LB) segregated from descendants of crosses with Asominori; however, the cause of this cosegregation was unclear. In 2004, we obtained five Asominori backcrossed inbred lines (BILs). Firstly, we evaluated the resistance to LB and BLB in these BILs. The partial resistance to LB was high, similar to criterial varieties such as Ouu320 and Chubu45. The BILs were also resistant to the BLB strain T-7174 (race1). Using 21 PCR-based DNA markers, we analyzed the genotypes of the BILs on chromosome 4, where BLBR and *Ph* genes are located. All five BILs carried a common Asominori-derived chromosomal segment estimated to be 1336 kbp long. Secondly, segregation of BLBR and LBR was monitored using 118 F_3 lines obtained from a cross between Ukei854 and Hitomebore, a BLB- and LB-susceptible cultivar. Most resulting F_3 lines showing BLBR also carried LBR; however, several lines showed only BLBR or BLR, indicating that BLBR and LBR were controlled by very closely linked loci. Using RM5473-2, we surveyed the genotypes of the 118 F_3 lines located on the common segment. Most F_3 lines with a high LBR showed genotypes identical to Asominori. Thus, the LBR locus in Asominori was tightly linked to the BLBR locus on chromosome 4, and the LBR gene was probably located near RM5473-2.

Keywords Asominori · Bacterial leaf blight · Leaf blast · Partial resistance · *Ph* gene

1 Introduction

Rice blast caused by *Magnaporthe grisea* is the most serious disease encountered in rice production. Rice plants (*Oryza sativa* L.) are often exposed to a

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cool temperature at the booting stage in the Tohoku region, northeastern area of Japan; this causes male sterility and accelerates the blast disease. It results in a devastating loss in yield of rice production. Therefore, one of the most important purposes of rice breeding is to improve the resistance of rice plants to blast disease.

A Japanese rice cultivar Asominori has been studied for its high resistance to bacterial leaf blight (BLB) caused by *Xanthomonas oryzae* pv. *oryzae* (Matsumoto et al. 1977, Sato et al. 1977, Sato 1978). Asominori has a high-resistance gene against BLB, *Xa1-as*(t) that shows high levels of resistance against both BLB race1 and race5. *Xa1-as*(t) is linked with the *Ph* gene located on chromosome 4 (Ise 1998). The *Ph* gene induces a brown reaction on the grain surface when the grain is soaked in phenol solution. We developed backcrossed inbred lines (BILs) derived from a cross using Asominori, which has a high partial resistance to leaf blast (LB) (Yokogami and Kato 2002). Some Japanese rice breeders noticed that the descendants of a cross with Asominori have resistances against both BLB and LB (Ogawa and Yagi 1992). However, the cause of this cosegregation was unclear. Hence, the objectives of this study are to clarify the relationship between bacterial leaf blight resistance (BLBR) and leaf blast resistance (LBR) and to develop a DNA marker tightly linked to the LBR gene.

2 Materials and Methods

2.1 Plant Materials

Five BILs and 118 F_3 lines were used for this study. Firstly, we carried out a cross between Okiniiri and Asominori in 1997 and a backcross between the F_1 plant and Okiniiri, which was used as a recurrent parent in 1998. Okiniiri is a cultivar with a slightly high partial resistance to LB and susceptibility to BLB. After 1998, individuals and lines were selected based on the resistance to LB. Consequently five BILs—Ukei851, Ukei852, Ukei854, Ukei856, and Ukei857—were obtained in 2004.

Secondly, we developed F_3 lines derived from F_2 individuals for genetic analysis. A cross between Ukei854 and Hitomebore was conducted in 2004. Ukei854 has a relatively high partial resistance to LB. On the contrary, Hitomebore is susceptible to both LB and BLB. Further, 118 F_2 individuals were selected at random, and 118 F_3 lines derived from F_2 individuals were developed in 2005.

2.2 Evaluation for LBR and BLBR

Both LBR and BLBR were evaluated at the National Agricultural Research Center for Tohoku Region (NARCT), Daisen, Akita, Japan, in 2006.

To evaluate a partial resistance to LB, the seeds of the five BILs, 118 F_3 lines, and the parent cultivars were sown in an upland nursery with two replications. Approximately 100 seeds were drilled in a one-row plot that was 35-cm long with

8-cm row spacing. Inabawase, a variety susceptible to LB, was sown on both sides of the plot area to enhance the natural inoculum. Disease severity was evaluated at 8–10 weeks after sowing by using a visual assessment method; scores ranging from 0 (no symptom) to 10 (all leaves dead) were assigned based on the diseased leaf area (Asaga 1981).

To evaluate the resistance to BLB, the seedlings of the five BILs, 118 F_3 lines, and parent cultivars were transplanted in a paddy field with two replications. Five seedlings were transplanted in one plot with an interval of 11.1 seedlings (m^{-2}). The clipping method was employed for the inoculation of BLB (Ogawa and Sekizawa 1980). The tip of a flag leaf was cut using scissors drenched with a bacterial suspension at one week before the heading date. The inoculated BLB strain was T-7174 (race1), which is incompatible with Asominori. To increase the BLB infection, nitrogen fertilizer was applied at the rate of 20 kg $N.ha^{-1}$ three times as a dressing before the heading date. Depending on whether the inoculated leaves cause chlorosis, disease severity was classified into three types by visual assessment at three weeks after inoculation. Thus lines with chlorosis were considered susceptible, lines without chlorosis were considered resistant, and lines that exhibited segregation of disease severity were considered segregated.

2.3 Analysis of Genotypes in BILs and F_3 Lines

DNA was extracted from the leaves by using the CTAB method described by Murray and Thompson (1980). For the analysis of the genotypes in the BILs, we used 21 PCR-based markers, including 16 simple sequence repeat (SSR) markers, 3 cleaved amplified polymorphic sequences (CAPS) markers, and 2 sequence tagged site (STS) markers (primer sequence not shown). One SSR marker (YX4-1), three CAPS markers (YX4-2, YX4-3, and YX4-4), and two STS markers (YX4-5, NX4-1) were developed using the sequence data of Asominori or using the Rice DNA polymorphism database (Laboratory for Plant Gene Function). All DNA markers were amplified under the following conditions. One cycle at 94 °C for 4 min; followed by 35 cycles at 94 °C for 1 min, 55 °C (50 °C or 60 °C) for 1 min, and 72 °C for 2 min; and finally one cycle at 72 °C for 7 min. In case of SSR and STS markers, amplified DNA products were electrophoresed in 3% (w/v) agarose gel (Sigma-Aldrich, St. Louis, MO) or 3% (w/v) Metaphor agarose gel (Cambrex, East Rutherford, NJ). When three CAPS markers were applied, the PCR products were digested by restriction enzymes (Hae3, Hha1, and Msp1). The digestion products were analyzed on 2% (w/v) agarose gels.

3 Severity of LB and BLB in BILs of Asominori

Figure 1 shows the score of LB severity in BILs, parental cultivars, and criterial cultivars. The scores of disease severity in Asominori, Ukei854, and the other BILs were 3.7, 3.5, and 2.8 respectively. On the other hand, the scores in Okiniiri

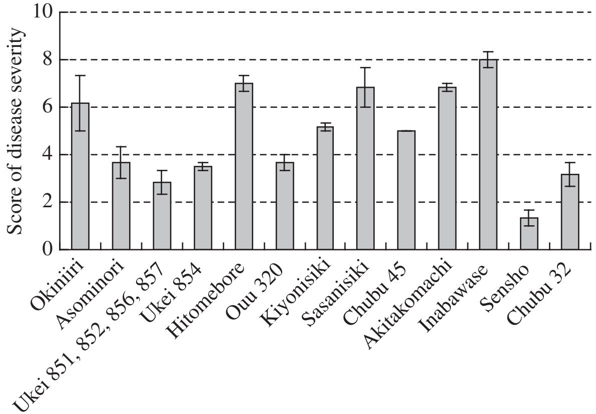

Fig. 1 Score of leaf blast severity in backcrossed inbred lines. The disease severity was scored using a scale ranging from 0 (*highly resistant: no symptom*) to 10 (*highly susceptible: all leaves dead*). The lines on the bars indicate standard error. The complete resistance genes of BILs, parental cultivars, and representative varieties with a high level of partial resistance are shown *bellow*. Ukei851 (*Pia*), Ukei852 (*Pia, Pii*), Ukei854 (*Pia, Pii*), Ukei856 (*Pia, Pii*), Ukei857 (*Pia, Pii*), Okiniiri (*Pia, Pii*), Asominori (*Pia*), Hitomebore (*Pii*), Sensho (*Pia*), and Chubu32 (*Pik-s*). The complete resistance and partial resistance levels of the criterial varieties are shown as follows. Ouu320 (*Pia, very high*), Kiyonishiki (*Pia, slightly high*), Sasanisiki (*Pia, slightly low*), Chubu45 (*Pii, high*), Akitakomachi (*Pii, Pia, moderate*), and Inabawase (*Pii, low*)

(a recurrent cultivar in BILs) and Hitomebore (a parent in F_3 lines) were 6.2 and 7.0, respectively.

In the Tohoku region, northeastern area of Japan, there are only limited predominant fungus races such as 007 and 037, and these races are compatible with complete resistance genes, namely, *Pia*, *Pii* and *Pik* (Zenbayashi et al. 2002). From this viewpoint, criterial varieties were used to evaluate the level of partial resistance to LB (Kataoka et al. 2004). They were firstly classified into three groups based on their complete genes: group A (*Pik-s*, *Pia*), group I (*Pii*, *Pia* + *Pii*) and group K (*Pik*, *Pia* + *Pik*) and then seven classes according to their level of partial resistance—very high, high, slightly high, moderate, slightly low, low, and very low on each group. Complete resistance genes of the five BILs and parental cultivars were estimated using the simplified method described by Tamura et al. (1995). We found that Asominori and Ukei851 contained *Pia*; Okiniiri, Ukei852, Ukei854, Ukei856, and Ukei857 contained *Pia* and *Pii*; and Hitomebore had *Pii*.

Among the criterial varieties, the score of disease severity in Ouu320, which has *Pia* and ranked as very high resistance, was 3.7 and that of Chubu45, which has *Pii* and high resistance, was 5.0. Furthermore in representative very-high-resistance varieties, the scores of disease severity of Chubu32 with *Pik-s* and Sensho with *Pia* were 3.2 and 1.3, respectively. Compared to these varieties, the score of disease severity in BILs was approximately identical or less, although it was higher than the disease severity of Sensho. Thus, the partial resistance to LB of BILs was ranked as very high resistance.

The BLB severity was estimated in BILs and Hitomebore. Chlorosis was not observed in Asominori and the five BILs. On the other hand, the tip of the leaves in Hitomebore clearly exhibited chlorosis (data not shown). Therefore, we found that Asominori and BILs were resistant and Hitomebore was susceptible to the BLB strain, T-7174 (race1).

4 Graphical Genotype of BILs

Figure 2 shows a graphical genotype on chromosome 4 in BILs. Based on the fact that Asominori has the BLBR gene, *Xa1-as*(t), associated with the *Ph* gene located on chromosome 4, the genotype of BILs was analyzed using 21 PCR-based DNA

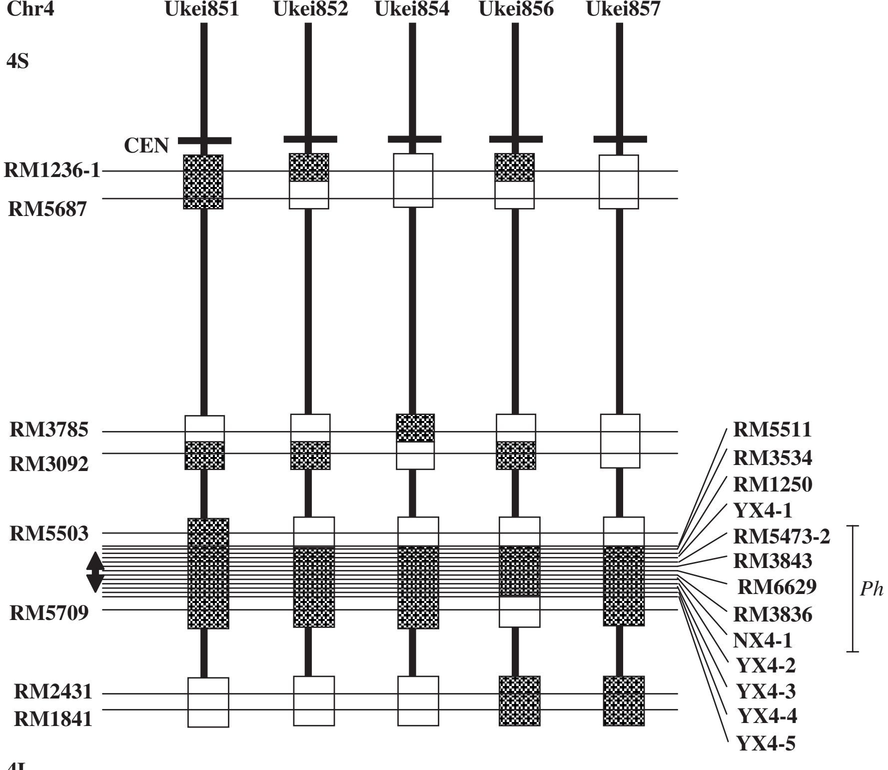

Fig. 2 Graphical genotype of backcrossed inbred lines. Different columns represent the following. □: Homozygote of Hitomebore, ■: Homozygote of Asominori. The arrow indicates a common region containing the chromosomal segment of Asominori in the backcrossed inbred lines—Ukei851, Ukei852, Ukei854, Ukei856, and Ukei857. The putative Ph gene locus is shown based on the physical map of Nipponbare and the phenotype of the recombinants. CEN, 4L, and 4S indicate the centromere, long arm, and short arm on chromosome 4, respectively

markers on chromosome 4. As a result, a common chromosomal segment of Asominori was detected between RM5511 and YX4-5 in the five BILs. The common chromosomal segment was located on the long arm on chromosome 4, and its size was estimated to be 1336 kbp by the physical map of Nipponbare, *Oryza sativa* ssp. japonica (Gramene database). The putative *Ph* gene locus based on the physical map of Nipponbare and the phenotype of recombinants (data not shown) is also shown in Fig. 2. Thus, these results implied that both the LBR and BLBR genes of Asominori are probably located on this common chromosomal segment.

5 Frequency Distribution of the Leaf Blast Severity in the F_3 Lines

The severity of both LB and BLB were evaluated in the F_3 lines derived from a cross between Ukei854 and Hitomebore. Figure 3 shows the frequency distribution of the score of LB severity. A variation in LB severity was observed continuously in the F_3 lines. The scores of LB severity in Ukei854 and Hitomebore were 3.5 and 7.0, respectively, and there was a significant ($P < 0.05$) difference between the parental cultivars. In the Figure 3(a), each line was classified into three types—resistant line, segregated line, and susceptible line—based on the BLB severity. With regard to the scores of F_3 lines, the mean score of the lines susceptible to BLB was 6.04 while that of the lines with resistant to BLB was 4.04; the difference between the mean scores of the two types was significant ($P < 0.01$).

Figure 3(b) also shows the frequency distribution of the leaf area with LB in the F_3 lines. Each line was classified into three types—homozygote of Asominori, heterozygote, and homozygote of Hitomebore—according to the genotype on the RM5473-2 locus on chromosome 4 in the F_2 individuals, which are the past generation of the F_3 lines. RM5473-2 is one of the DNA markers detected on the common chromosomal segment of Asominori in the BILs. With regard to the score of the F_3 lines, the mean score of Hitomebore-homozygote lines was 6.06 while that of Asominori-homozygote lines was 3.89; The difference between the mean scores of the two types was also significant ($P < 0.01$). Consequently, it was found that the LBR gene of Asominori was tightly linked to the BLBR gene, *Xa1-as(t)*, and the DNA marker, RM5473-2.

The F_3 lines were classified into two groups based on the score of LB severity; the R (resistant) group with a score of 5.5 or less and the S (susceptible) group with a score of more than 5.5. The segregation ratio of the number of lines in the R group to that in the S group was 91:27; it corresponded to the expected ratio of 3:1 (R:S), as revealed by the chi-square test ($P < 0.01$). Therefore, it is suggested that the partial resistance in Asominori is controlled by a single dominant gene. Currently, it has been reported that some partial resistance genes are controlled by a single dominant gene such as *Pi34* (Zenbayashi-Sawata et al. 2007) or *Pi35(t)* (Nguyen et al. 2006). A QTL locus and gene conferring a partial resistance to LB at the long arm on chromosome 4 was also reported (Miyamoto et al. 2001, Hirabayashi

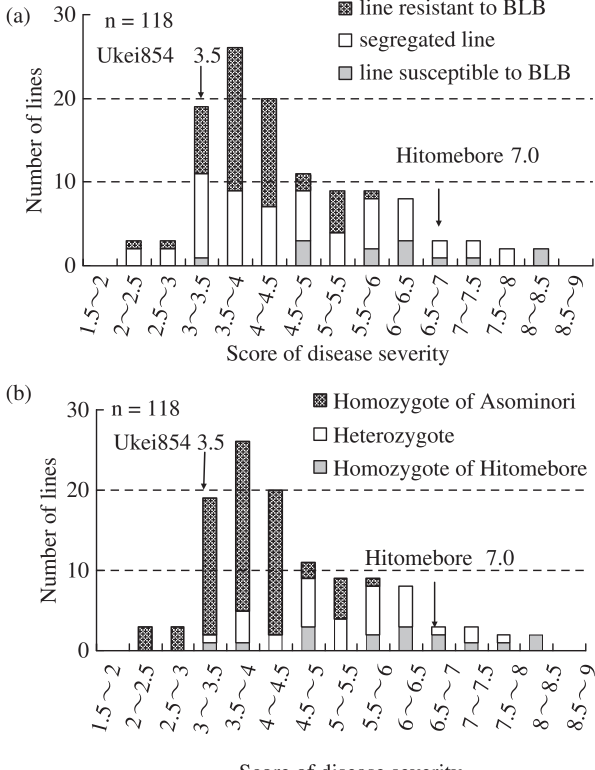

Fig. 3 The frequency distribution of leaf blast severity in F_3 lines. The LB disease severity was scored using a scale ranging from 0 (*highly resistant: no symptom*) to 10 (*highly susceptible: all leaves dead*). The *arrow* indicates the score of disease severity in the parental cultivars. (**a**) Different colors of the columns indicate different bacterial leaf blight (BLB) severity. *Black, white, and gray* columns represent the BLB-resistant lines, segregated lines and BLB-susceptible lines, respectively. (**b**) Different colors of columns indicate different genotypes on RM5473-2 in F_2 individuals, which are the past generation of the F_3 lines. *Black, white, and gray* columns represent the lines of the homozygote of Asominori, the heterozygote, and homozygote of Hitomebore, respectively

et al. 2005, Terashima et al. 2006). Accordingly, whether or not the LBR gene of Asominori is identical to these genes should be confirmed in the future.

Log likelihood (LOD) was calculated by Mapmaker/QTL ver1.1b (Lincoln et al. 1993) to examine the linkage of the LBR gene with the two loci, *Xa1-as(t)* and RM5473-2. Similarly, the additive effect and variation were calculated to analyze the genetic effects. The LOD scores for the *Xa1-as(t)* and RM5473-2 loci were 6.355 and 15.708, respectively (Table 1). These scores were greater than 2.0, which is the threshold value used to detect the putative QTL locus in interval mapping. This fact verified that these loci were tightly linked with the LBR gene. The additive effects were estimated to be −1.01 and −1.09, respectively. This implies that the score

Table 1 Linkage and genetic effects on *Xa1-as(t)* and RM5473-2 loci

Locus	LOD	Additive effect	Variation
Xa1-as(t)	6.355	−1.007	22.0
RM5473-2	15.708	−1.091	45.8

Log likelihood (LOD), additive effect, and variation were calculated by MAPMAKER/QTL Ver1.1b (Lincoln et al. 1993).

of disease severity decreased to 2.0 when the chromosomal segment substituted Asominori for Hitomebore on these loci. For example, if the score of disease severity changed from 4 to 6, it will correspond to 11.4%–44.3% of diseased leaf area; this is calculated by the formula (Asaga 1981), $\log\ y/1-y = 0.36873\times-2.364370$, where $y \times 100 = \%$ diseased leaf area and $x =$ score of disease severity. It is considered that the LBR gene of Asominori plays a role in the improvement of resistance to LB, because 32.9% decrease in the disease leaf area is efficient. The variation in the *Xa1-as(t)* and RM5473-2 loci displayed 22.0% and 45.8%, respectively. In particular, the RM5473-2 locus explained approximately 45.8% of the total phenotypic variation in the F_3 lines. Hence, the LBR gene was expected to be located near the position of the marker RM5473-2.

6 Further Study

Importantly, the rice breeding strategy of Japan should include diversified genetic resources such as rice varieties from foreign countries, upland rice, and wild rice as a parent of a cross to improve the partial resistance to LB. Simultaneously, it is more important to pyramid the resistance genes without undesirable linkages, such as vivipary, long culm length, bad grain quality, bad eating quality, and low yield. Therefore, any linkage between a resistance gene locus and an agriculturally important trait must be clarified. Furthermore, the resistance genes are important in demonstrating the interaction with other LBR genes and evaluating the effect of the recipient (parent of the cross).

The LBR gene of Asominori is characterized by a close linkage with BLBR. Introduction of both the LBR and BLBR genes simultaneously in a new variety may be possible by using DNA markers. In various aspects, Asominori is similar to the typical Japanese rice varieties in plant type, but its ancestor is a Filipino rice variety, Taducan. From this viewpoint, exploring the origin of the LBR gene in Asominori is an interesting topic of research.

In a future study, we would like to identify the position of the LBR gene and develop a practically perfect marker to identify LB resistant lines. Moreover, we are planning to develop a Hitomebore, which is a leading cultivar in the Tohoku region, near isogenic line carrying the LBR gene by using a practically perfect marker.

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DNA Marker Analysis of Blast Resistance Genes *Pib* and *Pita* in IRRI-Bred Rice Varieties Comparing with Gene Estimation by a Differential System

Daisuke Fujita, Leodegario A. Ebron, Nobuya Kobayashi and Yoshimichi Fukuta

Abstract Blast resistance genes are important in rice (*Oryza sativa* L.) improvement programs. The DNA markers linked to resistance genes are a powerful tool to detect the presence of genes and are widely used to select breeding materials through marker-assisted selection. This study was conducted to evaluate the detection ability of DNA markers for rice blast resistance genes *Pib* and *Pita* in IRRI-bred rice varieties. Forty-two Indica-type varieties, which have been previously analyzed for the presence of *Pib* and *Pita* by conventional genetic analysis using a differential system involving standard blast isolates (*Pyricularia grisea* Sacc.) from the Philippines, were tested. To estimate the presence of *Pib* and *Pita*, previously reported PCR-based dominant markers were used. The target DNA fragments of *Pib* using Sub3-5 were amplified in 40 varieties but not in two varieties. Also, the target DNA fragments of *Pita* using three gene-specific markers were amplified in 27 or 28 varieties but not in 14 or 15 varieties. The results of DNA marker analysis of the 42 IRRI-bred rice varieties were almost the same as those of previous gene estimation of *Pib* and *Pita* by the differential system. It suggests that the efficiency of detecting blast resistance genes through use of DNA markers depends on the rice variety and the DNA markers. The proper markers for the *Pita* gene provide a basis for stacking other blast resistance genes into high-yielding and good-quality advanced breeding rice lines.

Keywords DNA marker · IRRI-bred rice variety (*Oryza sativa* L.) · *Pib* · *Pita* · Resistance gene

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1 Introduction

Blast resistance genes are important in rice (*Oryza sativa* L.) improvement programs. The resistance to blast disease is governed by a gene-for-gene relationship between the resistance gene in the host and the avirulence gene in the blast pathogen *Pyricularia grisea* (Cooke) Sacc. (Silue et al. 1992). Around 40 blast resistance genes have been identified by genetic studies and five resistance genes- *Pib*, *Pita*, *Piz-5*(*Pi2*), *Piz-t*, and *Pi9*- have been isolated (Wang et al. 1999; Bryan et al. 2000; Qu et al. 2006; Zhou et al. 2006). Two major resistance genes, introgressed from Indica cultivars, *Pib* and *Pita*, encoded predicted nucleotide-binding site type proteins (Wang et al. 1999; Bryan et al. 2000). Specific gene markers are a powerful tool to detect the presence of genes and are widely used to select breeding materials through marker-assisted selection. Several gene-specific markers of *Pib* and *Pita* have been developed using the genome sequence of these genes in resistant and susceptible varieties (Wang et al. 1999; Jia et al. 2002; Fjellstrom et al. 2004).

Genetic studies of blast resistance were limited by the lack of a suitable differential system for blast resistance genes. The 12 Japonica-type differential varieties for blast resistance were selected by Yamada et al. (1976) and Kiyosawa (1981), but they carried additional gene(s) to tropical blast isolates, which masked the targeted gene's reaction (Noda et al. 1999). To overcome this limitation, the pathogenicities of blast isolates from the Philippines were clarified using differential varieties and lines (Yanoria et al. 2000). Several blast isolates with distinct pathogenicities were selected and studied in detail using a set of monogenic lines that contains 23 kinds of single resistance genes with the genetic background of a Chinese variety Lijiang-xin-tuan-heigu (Tsunematsu et al. 2000). Using this system, a total of seven resistance genes- *Pi20, Pita, Pik*† (one of *Pik* alleles, *Pik*, *Pik-h*, *Pik-m*, or *Pik-p*), *Pib*, *Pik-s*, *Piz-t,* and *Pii* or *Pi3*(t)- in 42 IRRI-bred rice varieties were estimated and 42 IRRI-bred varieties were classified into seven variety groups mainly on the basis of the presence of *Pi20*, *Pita*, and one of *Pik*† allele genes (Ebron et al. 2004). To confirm the presence of resistance genes in IRRI-bred varieties, conventional genetic analyses (segregation analysis and allelism test) were performed using BC_1F_2 and F_2 populations, respectively (Ebron et al. 2005).

During the 1970s and up to the 1980s, many IRRI-bred varieties have been released and distributed worldwide and used by farmers and breeders as important parental varieties in breeding programs. IR8, which was released in 1966, triggered the green revolution in tropical countries of Asia (Hossain 1995). In the 1970s, IR36 had been grown widely in several Asian countries. IR64, which was released in 1985, had been widely accepted as a high-quality rice variety in many countries (Khush 1987). A more recent variety, IR72, shows a high yield potential, shorter growth duration, and enhanced resistance to several diseases and insect pests. IRRI-bred varieties and their progenies are now planted in many of the world's rice areas. It is important to understand blast resistance genes in IRRI-bred varieties for rice breeding.

In this study, the detection ability of gene-specific markers for *Pib* and *Pita* were evaluated. Forty-two IRRI-bred varieties were analyzed using four PCR-based

dominant markers that were designed on the basis of sequence information of blast resistance genes *Pib* and *Pita*. The results from the detection of DNA fragments by PCR were compared with those obtained from gene estimation of *Pib* and *Pita* by conventional genetic analysis.

2 Materials and Methods

2.1 IRRI-Bred Rice Varieties

A total of 42 Indica-type varieties at IRRI, which have been previously analyzed by conventional genetic methods, were used. Thirty-four of these 42 Indica-type varieties are IR varieties, which include IR8, IR24, IR36, IR64, and IR72. They have been widely distributed and used in many countries. Eight varieties- PSBRc1, PSBRc2, PSBRc4, PSBRc10, PSBRc18, PSBRc20, PSBRc28, and PSBRc30- were developed by IRRI and designated by the Philippine Seed Board. Six other varieties- Nipponbare, Yashiro mochi, BL1, IRBLb-B (monogenic line for *Pib* derived from BL1), K1, and IRBLta-K1 (monogenic line for *Pita* derived from K1)- were used as controls. The 42 IRRI-bred varieties were classified into seven variety groups, VG 1 to VG 7, based on a differential system (Table 1). In some cases, subgroups within a group were also identified because the variety reacted differentially to a particular isolate. VG 1, VG2, and VG 7 were further divided into two, three, and two subgroups, respectively.

2.2 DNA Markers and Genotyping

Genomic DNAs were extracted from fresh leaves using the CTAB method (Murray and Thompson 1980). The primer set for *Pib*, Sub3-5 (5′-AGGGAAAAATGGA AATGTGC-3′ and 5′-AGTAACCTTCTGCTGCCCAA-3′), was used to detect *Pib*, and primer sets for *Pita*, $Pita_{440}$ (YL153: 5′-CAACAATTTAATCATACACG-3′ and YL154: 5′-ATGACACCCTGC-GATGCAA-3′), $Pita_{1042}$ (YL155: 5′-AGCAGGTT ATAAGCTAGGCC-3′ and YL87: 5′-CTACCAACAAGTTCATCAAA-3′), and $Pita_{403}$ (YL100: 5′-CAATGCCGAGTGTGCAAAGG-3′ and YL102: 5′-TCAGGTT GAAGATGCATAGC-3′) were used for *Pita* according to previous reports (Wang et al. 1999; Jia et al. 2002). The genotypes of *Pib* and *Pita* using these primers were then determined by PCR amplification in a DNA engine dyad Peltier thermal cycler (Bio-Rad). The 15 μl PCR reaction mixture contained 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 200 μM dNTP, 0.2 μM primer, and 5–10 μg/ml of genomic DNA as template. The thermal cycler was programmed for a first denaturation step of 5 min at 95 °C, followed by 35 cycles, each of 30 s at 95 °C, 30 s at 55 °C for three markers, Sub3-5, $Pita_{440}$ (YL153/YL154), and $Pita_{1042}$ (YL155/YL87), and at 64.5 °C for $Pita_{403}$ (YL100/YL102) and 30 s at 72 °C. The SSR products

Table 1 Gene identification by conventional genetic analysis of two resistance genes *Pib* and *Pita* in 42 IRRI-bred varieties

Variety group	IRRI-bred vairety	*Pib*			*Pita*		
		Reaction pattern[1]	BC_1F_2 analysis[2]	Allelism test[2]	Reaction pattern[1]	BC_1F_2 analysis[2]	Allelism test[2]
VG 1a	IR20, IR28, IR30, IR45, IR66	+			–		
VG 1b	IR29, IR34	+	+ (IR34)	+ (IR34)	–		
VG 2a	IR8, IR22, IR24, IR26, PSBRc30	+	+ (IR24)	+ (IR24)	–		
VG 2b	IR43	+			–		
VG 2c	PSBRc2	+			–		
VG 3	IR5, IR32, IR36, IR38, IR40, IR42, IR44, IR50, IR52, IR54, IR58, IR60, IR62, IR65, IR68, IR72, PSBRc4	+	+ (IR36, IR60)	+ (IR36, IR60)	+	+ (IR36, IR60)	+ (IR36)
VG 4	PSBRc1	Unknown			–		
VG 5	IR74	Unknown	+		–		
VG 6	IR56, IR70	Unknown			+		+
VG 7a	IR46, IR48, IR64, PSBRc28	+	+ (IR46, IR64)	+ (IR64)	+	+ (IR46, IR64)	+ (IR64)
VG 7b	PSBRc10, PSBRc18, PSBRc20	+			+		

[1] Classification of IRRI-bred varieties and genes *Pib* and *Pita* estimated using a differential system involving Philippine blast isolates of *P. grisea* modified from the data of Ebron et al. (2004).
[2] Classification of IRRI-bred varieties and genes *Pib* and *Pita* estimated using genetic analysis and allelism test using data of Ebron et al. (2005).
+ or – indicates presence or absence of genes *Pib* and *Pita*, respectively.

were resolved in 1.0% agarose gel by electrophoresis at 200 V for 1 h in 0.5 X TBE buffer. The gels were stained with ethidium bromide and photographed under ultraviolet light.

3 Results

3.1 Genotyping of Pib *and* Pita *Using DNA Markers*

BL1 and IRBLb-B that carry *Pib* were used as positive control, while Yashiromochi and Nipponbare were used as negative control. The presence and absence of *Pib* genes in 42 IRRI-bred rice varieties and control varieties were confirmed using DNA marker, Sub3-5 (Table 2). The DNA fragments of the positive control varieties were amplified, whereas those of negative control were not. For 42 IRRI-bred rice varieties, DNA fragments using Sub3-5 were amplified in 40 varieties, while DNA fragments of two varieties, PSBRc18 and IR54, were not amplified. Similarly, K1, IRBLta-K1, and Yashiromochi carrying *Pita* were used as positive control, while Nipponbare was used as the negative control. The presence and absence of the *Pita* genes in 42 IRRI-bred rice varieties and control varieties were confirmed using three DNA markers, $Pita_{440}$ (YL153/YL154), $Pita_{1042}$ (YL155/YL87), and $Pita_{403}$ (YL100/YL102) (Table 3). The DNA fragments of the positive control varieties were amplified, while those of the negative control were not. For 42 IRRI-bred rice varieties, DNA fragments using markers, $Pita_{440}$ and $Pita_{1042}$ were detected in 28 varieties, while DNA fragments of 14 varieties were not amplified. Also, DNA fragments using $Pita_{403}$ were amplified in 27 of the 42 IRRI-bred rice varieties, while DNA fragments of 15 varieties were not amplified.

3.2 Comparison Between Conventional Genetic Analysis and DNA Marker Analysis

The detection of DNA fragments for *Pib* in the positive and negative control varieties were compared in terms of the presence and absence of *Pib*. The DNA fragments of Sub3-5 in positive control varieties BL1 and IRBLb-B that carry *Pib* were detected, whereas those of negative control varieties Yashiromochi and Nipponbare were not. For control varieties, the results of DNA fragments were completely matched in terms of presence and absence of *Pib*. The detection of DNA fragments for *Pib* in 37 IRRI-bred rice varieties gave results similar to those obtained gene estimation of *Pib* using conventional genetic analysis. Results in two varieties were different from the gene estimation results for *Pib* (Table 2). Although these two varieties, IR54 and PSBRc18, were estimated to carry *Pib* using conventional genetic analysis, these varieties had no DNA fragments for *Pib* detected. Three varieties-IR56, IR70, and PSBRc1- were not tested as to whether they carry *Pib* using the differential system. Therefore, the results with respect to DNA fragments for *Pib*

Table 2 Gene identification by conventional genetic analysis and DNA marker of *Pib* in 42 IRRI-bred varieties

		IRRI-bred variety																																										
		1a[1]					1b		2a					2b	2c	3																	4	5	6		7a				7b			Control		
Gene	Method of gene identification	IR20	IR28	IR30	IR45	IR66	IR29	IR34	IR8	IR22	IR24	IR26	PSBRc30	IR43	PSBRc2	IR5	IR32	IR36	IR38	IR40	IR42	IR44	IR50	IR52	IR58	IR60	IR62	IR65	IR68	IR72	PSBRc4	IR54	PSBRc1	IR74	IR56	IR70	IR46	IR48	IR64	PSBRc28	PSBRc10	PSBRc20	PSBRc18	BL1	IRBLb-B	Yashiromochi
Pib	Conventional analysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	/	+	/	/	+	+	+	+	+	+	+	+	+	−
	Sub3-5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−

[1] Number refers to variety group based on differential system.
+ or − indicates presence or absence of *Pib*, respectively. Slash (/) means unknown data from reaction patterns.

Table 3 Gene identification by conventional genetic analysis and DNA markers of resistance gene + in 42 IRRI-bred varieties

Method of gene identification	IRRI-bred variety																																										Control			
	1a[1]					1b		2a					2b	2c	3																	4	5	6		7a				7b						
	IR20	IR28	IR30	IR45	IR66	IR29	IR34	IR8	IR22	IR24	IR26	PSBRc30	IR43	PSBRc2	IR5	IR32	IR36	IR38	IR40	IR42	IR44	IR50	IR52	IR54	IR58	IR60	IR62	IR65	IR68	IR72	PSBRc4	PSBRc1	IR74	IR56	IR70	IR46	IR48	IR64	PSBRc28	PSBRc10	PSBRc18	PSBRc20	KI	IRBLta-KI	Yashiromochi	Nipponbare
Conventional analysis	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	−
$Pita_{440}$	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	−
$Pita_{1042}$	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	−
$Pita_{403}$	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	−

[1] Number refers to variety group based on differential system.
+ or − indicates presence or absence of *Pita*, respectively.

in 42 IRRI-bred varieties were mostly the same as those of gene estimation using conventional genetic analysis.

The DNA fragments of the positive and negative control varieties for *Pita* were compared with the presence and absence of *Pita*. The DNA fragments of $Pita_{440}$, $Pita_{1042}$, and $Pita_{403}$ in positive control varieties K1, IRBLta-K1, and Yashiromochi, which carry *Pita*, were detected; those of negative control variety Nipponbare were not detected. For control varieties, the results of DNA fragment analysis completely matched those that determined the presence and absence of *Pita*. The detection of DNA fragments for *Pita* using two DNA markers, $Pita_{440}$ and $Pita_{1042}$, in 40 IRRI-bred rice varieties gave the same results as did gene estimation for *Pita* using conventional genetic analysis, except for two varieties (Table 3). Although PSBRc1 and PSBRc2 were estimated to be not carrying *Pita* by conventional genetic analysis, DNA fragments for *Pita* in these varieties were detected using $Pita_{440}$ and $Pita_{1042}$. The detection of DNA fragments for *Pita* using $Pita_{403}$ in 41 IRRI-bred rice varieties yielded the same results as those of gene estimation for *Pita* using conventional genetic analysis, whereas in one variety, a different result was found. Although PSBRc1 was predicted not to carry *Pita* by conventional genetic analysis, it had detected DNA fragments for *Pita*. Therefore, the results regarding DNA fragments for *Pita* in 42 IRRI-bred varieties were almost the same as those shown by gene estimation using conventional genetic analysis.

4 Discussion

The use of gene-specific markers enabled the detection of genes *Pib* and *Pita* in most IRRI-bred rice varieties, previously identified to be harboring these genes by the use of a differential system. The *Pib* gene was previously estimated in IR54 and PSBRc18 by the differential system (Ebron et al. 2004) but it could not be detected using DNA markers in this study (Table 2). For these varieties, further DNA analysis using other gene-specific markers for *Pib*, (e.g., Pibdom, which was developed by Fjellstrom et al. [2004]), will be needed. Although three varieties, IR56, IR70, and PSBRc1, were not estimated to have *Pib* by the differential system, DNA fragments using gene-specific markers were detected in them. In a previous analysis, IR56, IR70, and PSBRc1 were not identified to be carrying *Pib* using the differential system because of the narrow spectrum of resistance in *Pib*. *Pib* showed a narrow spectrum of resistance to blast isolates from the Philippines and this reaction pattern might be completely masked by the presence of $Pik^{\dagger}$. Therefore, the results of DNA analysis suggested that IR56, IR70, and PSBRc1 may harbor the *Pib* gene.

Similarly, the gene-specific markers $Pita_{440}$, $Pita_{1042}$, and $Pita_{403}$ were useful and effective for *Pita* gene identification because the results pertaining to DNA fragments in all IRRI-bred varieties (excluding PSBRc1 and PSBRc2) were the same as those given by conventional genetic analysis. Although DNA fragments of *Pita* in PSBRc1 and PSBRc2 were detected, the reaction pattern results using the differential system showed that PSBRc1 and PSBRc2 did not have the *Pita*

gene. Additionally, the allelism test between IR56 (carrying *Pita* and *Pik*†) and PSBRc1 (carrying *Pik*†) showed that PSBRc1 did not have the *Pita* gene. Thus, the genome sequence of primer sites on $Pita_{440}$, $Pita_{1042}$ and $Pita_{403}$ (markers made by Yashiromochi sequence) in PSBRc1 and PSBRc2 is almost similar to the Yashiromochi sequence. Other genome sequences of *Pita*, excluding these primer sites, may be different from that between Yashiromochi and the two varieties, PSBRc1 and PSBRc2. This difference is important to develop more efficient gene-specific markers than $Pita_{440}$, $Pita_{1042}$, and $Pita_{403}$.

The utility of gene-specific markers was demonstrated in this study, and gene estimations using DNA analysis confirmed findings of the differential system. IR74 was not identified to be carrying *Pib* using the differential system because this gene was completely masked by the presence of *Pik*†, but IR74 was identified to have *Pib* using segregation analysis (Table 1). The DNA fragments of gene-specific marker for *Pib* in IR74 were also detected. Although *Pib* in IR74 was not detected using the differential system, *Pib* genes were detected using DNA analysis. This result suggests that precise gene estimation may be conducted using DNA analysis and the differential system in the case of a narrow spectrum of resistance. Three resistance genes, *Pi2*, *Piz-t*, and *Pi9*, have been cloned and sequenced (Qu et al. 2006; Zhou et al. 2006). Based on this information, gene-specific markers for these genes will be developed and can be used to identify the genotypes of *Pi2, Piz-t*, and *Pi9*. Therefore, various varieties, which carry multiple resistance genes and show complex reactions, will be identified to have resistance genes using gene-specific markers.

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Genetic Characterization of Universal Differential Variety Sets Developed Under the IRRI-Japan Collaborative Research Project

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Abstract The IRRI-Japan Collaborative Research Project has developed four universal differential variety sets, monogenic lines (MLs) with a Japonica-type variety Lijianxintuanheigu (LTH) genetic background, and near-isogenic lines (NILs) with three different genetic backgrounds, LTH, an Indica type variety CO39, and a universal susceptible line, US-2. These lines targeted 24 resistance genes:*Pia, Pib, Pii, Pik, Pik-h, Pik-m, Pik-p, Pik-s, Pish, Pita*(*Pi4*)*, Pita-2, Pit, Piz, Piz-5*(*Pi-2*), *Piz-t, Pi1, Pi3, Pi5*(t), *Pi7*(t), *Pi9, Pi11*(t), *Pi12*(t), *Pi19* and *Pi20*(t). The MLs have been distributed to more than 15 countries. To characterize these genetic components and confirm the introgression of chromosome segments harboring resistance genes, the graphical genotypes were determined using 162 simple sequence repeat (SSR) markers distributed throughout the rice genome. The genotypes of the three sets of NILs were more uniform than those of MLs. Several introgression segments, which corresponded to the locations of blast resistance genes, were also confirmed. The genome restoration rates of 31 MLs to LTH ranged from 50 to 90.0%, averaging 77.3%. All LTH, CO39, and US-2 NILs were developed by backcrossing six times with each recurrent parent. The genome restoration rates of the parent in 34 LTH NILs ranged from 75.6 to 96.9% with an average of 90.6%, lower than the theoretical value of 99%. The 31 CO39 NILs showed greater than 90% genome restoration rate compared to the recurrent parent with an average of 97.3%. The genomic restoration rate of the 16 US-2 NILs closely resembled those of the recurrent parent, with frequencies ranging from 88.9 to 98.8% with an average of 94.6%. Genetic characterization of four universal differential variety sets was carried out using DNA markers. This information will be linked with resistance genes that are potentially very useful for marker-assisted selection (MAS) in rice breeding programs, since the differential varieties can be applied as genetic sources.

Keywords Resistance gene · Differential variety · Chromosomal component · Blast (*Magnaporthe grisea*) · Rice (*Oryza sativa* L.)

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1 Introduction

Blast, caused by *Magnaporthe grisea*, is one of the most destructive diseases of rice (*Oryza sativa* L.) and the use of resistant varieties is an efficient way of controlling this disease. Differential varieties are important for improving blast resistance and for pathological studies. The relationship between the host plant (resistance genes) and pathogen (avirulence genes) can be explained by the gene-for-gene theory (Flor, 1971). Therefore, virulence genotypes of pathotypes can be inferred when resistance genotypes are known for each differential variety and these can distinguish pathotypes (races) based on their reaction pattern or qualitative differences in reactions to different pathogen strains. In Japan, sets of 9 varieties (Yamada et al., 1976) and 12 varieties (Kiyosawa, 1984) were selected and these have been used as blast differentials. However, these varieties were not readily available in other countries because several of them contained not only the target resistance genes but also harbored other resistance gene(s).

Near-isogenic lines (NILs) for blast resistance were developed for only four resistance genes, *Pi1, Pi3, Piz-5*, and *Pita*(*Pi4*) with the genetic background of an Indica-type rice, CO39, and these were used as differential varieties (Mackill et al., 1985; Mackill and Bonman, 1992). A set of NILs with a susceptible Japonica type variety Lijiangxintuanheigu (LTH) genetic background targeting six resistance genes, *Pita*, *Pita-2*, *Pib*, *Pik*, *Pik-m*, and *Pik-p*, was developed by Ling et al. (1995). However, the number of resistance genes covered by these NILs was limited.

To provide more useful materials than have been developed to date, the IRRI-Japan Collaborative Research Project released the MLs for blast resistance as the first set of international standard differential varieties (Tsunematsu et al., 2000). These MLs were developed for targeting 24 resistance genes, *Pia, Pib, Pii, Pik, Pik-h, Pik-m, Pik-p, Pik-s, Pish, Pita, Pita-2, Pit, Piz, Piz-5*(*Pi-2*), *Piz-t, Pi1, Pi3, Pi5*(t), *Pi7*(t), *Pi9, Pi11*(t), *Pi12*(t), *Pi19*, and *Pi20*(t), and have been distributed to more than 15 countries by IRRI (Fukuta et al., 2004a). The project is developing NILs further with three different genetic backgrounds, LTH, an Indica type variety, CO39, and a universal susceptible line, US-2, which was derived from a cross between an Indonesian land race, Kencana, and an Indica-type variety, Takanari, for targeting these resistance genes (Fukuta et al., 2004b, Kobayashi et al., 2007).

The reaction pattern of these differential varieties to the standard differential blast isolates from Japan and the Philippines, and their morphological and agronomical characters have also been studied. However, these studies have not yet been completed. Genotyping of these varieties using molecular markers is important to clarify the genetics of these differential varieties. Moreover, since differential varieties also have great potential to become genetic sources for breeding of blast resistance, availability of a molecular marker linked with the resistance gene is necessary for marker assisted breeding. This study has been carried out to clarify the genetic character of the differential varieties, and confirm the introgressed blast resistance genes using molecular markers. The development of differential varieties is reviewed and the prospects for further development under the IRRI-Japan Collaborative Research Project, and the utilization of differential varieties are discussed.

2 Materials and Methods

2.1 Development of Differential Variety

The blast resistance genes were introduced into three different genetic backgrounds by recurrent backcross breeding. Three susceptible varieties, LTH, CO39 and US-2 were used as the recurrent parents (Ando and Hayashi, personal communication). Twenty-five varieties were used as donor parents of blast resistance genes (Table 1).

A total of 20 Philippines strains, which were classified according to their pathogenicity and selected as the differential blast isolates (Yanoria et al., 2008), were used to study the reaction pattern of the resistance genes. Isolates that were avirulent

Table 1 Differential varieties developed by IRRI-Japan Collaborative Research Project

Target resistance gene	Chromosome[1]	No. of donor variety used	Differential varieties			
				NIL		
			ML	LTH	CO 39	US-2
Pit	1	1	1	–	–	–
Pish	1	4	2	–	5	–
Pib	2	3	1	1	2	–
Piz	6	1	1	–	–	–
Piz-5 (= *Pi2*)	6	3	2	3	1	–
Piz-t	6	2	1	1	1	1
Pi9	6	1	1	1	–	1
Pi11(t)	8	1	1	–	–	–
Pii	9	1	1	–	–	–
Pi3	9	1	1	2	–	–
Pi5(t)	9	1	1	1	1	1
Pia	11	3	2	3	–	2
Pik	11	2	1	1	2	1
Pik-s	11	5	2	3	1	2
Pik-p	11	1	1	–	1	1
Pik-h	11	1	1	1	1	1
Pik-m	11	1	1	–	1	–
Pi1	11	1	1	1	1	1
Pi7(t)	11	1	1	1	1	1
Pita (= *Pi4*)	12	5	3	3	1	3
Pita-2	12	4	2	2	3	–
Pi12(t)	12	1	1	–	–	1
Pi19	12	1	1	–	–	–
Pi20	12	1	1	–	–	–
Unknown	–	16	–	10	9	–
Total	–	62	31	34	31	16

[1] Chromosome locating resistance gene.
A total of resistance genes are targeted for the development of differential varieties.
Several donor varieties were used for each differential variety.
ML: monogenic lines, LTH: a Chinese Japonica-type variety, Lijianxintuanheigu. CO39: an Indica-type variety. US-2: a universal susceptible variety developed Indica and Japonica-types varieties' cross by Andoh and Hayashi (unpublished)

to the resistance genes were inoculated to confirm the presence and homozygosity of the corresponding genes in each developing progeny. The reaction was classified on a scale of 0–5 as described previously by Mackill and Bonman (1992), with slight modifications. Where the biggest lesion was elongated less than or more than 3 mm, the plant was rated as 3^- or 3^+, respectively. The reactions of differential varieties were categorized and summarized into three reaction classes wherein 0–2 were resistant (R), 3^- was moderately resistant (M), 3^+ was moderately susceptible (MS), and 4–5 were considered susceptible (S). LTH and CO39 were used as susceptibility validation varieties.

2.2 Characterization of Differential Variety

Agronomical traits including heading date and several morphological characteristics of the developed lines were evaluated to confirm the genetic uniformity and similarity to the corresponding recurrent parent.

To describe the genetic components of these differential varieties, the graphical genotype concept developed by Young and Tanksley (1989) was employed. A total of 162 simple sequence repeat (SSR) markers developed by McCouch et al. (2002) were used to develop graphical genotypes of the lines of all the differential varieties including MLs and the NILs of LTH, CO39 and US-2.

A simple DNA extraction method for PCR analysis (Wang et al., 1993) was used. The polymorphic bands were recorded for each line and compared with the banding pattern of recurrent parents LTH, CO 39, and US-2.

3 Results

Monogenic lines and three sets of NILs were developed as the differential varieties of rice blast by the recurrent backcross breeding method. The MLs targeted 24 blast resistance genes, *Pia, Pib, Pii, Pik, Pik-h, Pik-m, Pik-p, Pik-s, Pish, Pita* (*Pi4*), *Pita-2, Pit, Piz, Piz-5* (*Pi-2*), *Piz-t, Pi1, Pi3, Pi5*(t), *Pi7*(t), *Pi9, Pi11*(t), *Pi12*(t), *Pi19*, and *Pi20*(t) (Tsunematsu et al., 2000). The LTH NILs contained 15 rice blast resistance genes, *Pib*, *Piz-5*, *Piz-t*, *Pi3*, *Pi9*, *Pi5*(t), *Pia*, *Pik-s*, *Pik*, *Pik-h*, *Pi1*, *Pi7*(t), an unidentified gene, *Pita*, and *Pita-2*. The alleles in the NILs with *Pik** and *Pita** that showed the same reaction patterns to blast isolates from the Philippines to *Pik* and *Pita*, respectively, have not yet been confirmed. The CO39 NILs targeted 14 major rice blast resistance genes, *Pish, Pib, Piz-5, Piz-t, Pi5*(t), *Pik-s, Pik, Pik-h, Pik-m, Pik-p, Pi1, Pi7*(t), *Pita, Pita-2, Pik**, and *Pita**. The last set US-2 NILs contained 12 rice blast resistance genes, *Pia, Pik, Pik-h, Pik-p, Pik-s, Pi1, Pi7*(t), *Pita, Pi12*(t), *Piz-t, Pi9* and *Pi5*(t) (Table 1).

These MLs were designated as 'IRBL' lines (IRRI bred blast resistance lines) followed by the resistance gene, and then the abbreviation of the resistant donor variety. For example, IRBLa-A is a line with the resistance gene *Pia* originating from Aichi Asahi as the donor parent. These lines have been distributed to more

than 30 institutes around the world through the International Network for Genetic Evaluation of Rice (INGER) and the IRRI-Japan Collaborative Research Project, IRRI. In the case of NILs, the genetic backgrounds, LTH [LT], CO 39 [CO], and US-2 [US], were followed by the donor name.

3.1 Characterization of Agricultural Traits

When the heading date of the MLs and three types of NILs were evaluated, significant differences were observed among these differential variety sets, although they were completely fixed for the resistance to rice blast. The number of days to heading of LTH was 68 after transplanting in the paddy field, and the MLs varied from 60 to 120 days in Tsukuba city, Ibaraki, Japan. In the LTH, CO39, and US-2 NILs, it varied from 65 to 110 days, 65 to 70 days, and 76 to 84 days, respectively. These results indicated that the variation in each differential variety set changed according to the number of recurrent backcrosses and the type of parent. In addition, four differential varieties, IRBLzt-t, IRBLzt-T[LT], IRBLzt-IR56[CO], and IRBLzt-T[US] into which *Piz-t* was introduced, showed late heading in comparison with the other lines in the same variety set (Fig. 1). Yokoo et al. (1980) reported that a major photoperiod sensitive gene, *Se-1*, was located on chromosome 6 and linked with the

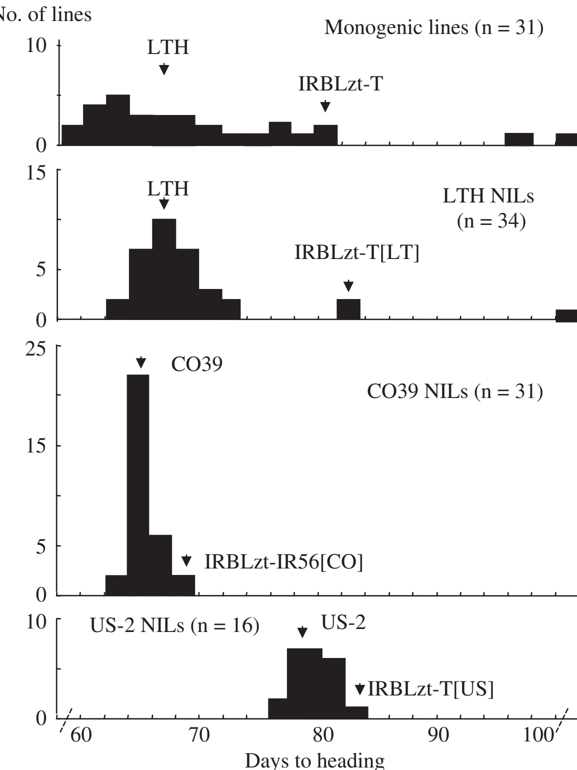

Fig. 1 Distributions of heading date in differential varieties at Tsukuba, Japan, in 2006. All lines introduced *Piz-t* showed late heading in comparison with the other lines

resistance gene, *Piz-t*. Therefore, these four lines might have the target resistance gene introduced together with the photoperiod sensitive gene.

3.2 Reaction Pattern of Differential Varieties

All lines in the differential variety sets were fixed with the resistance genes and showed clear reactions to 20 blast isolates from the Philippines. A comparison of the target resistance gene data in each donor variety with the differential varieties of various genetic backgrounds revealed that the results were consistent with the reaction results of the standard differential blast isolates (data not shown).

The total number of differential varieties, which showed the same reaction pattern in each target resistance gene and validated the non-introgression of any additional resistance gene, were 17, 23, 14, and 7 in MLs, LTH NILs, CO39 NILs, and US-2 NILs, respectively. The other lines, 14 in MLs, 11 in LTH NILs, 17 in CO39 NILs, and 9 in US-2 NILs, showed additional resistance reaction(s), and it is expected that some minor or other major resistance gene(s) might be harbored in these genetic backgrounds. For example, *Pish* was introgressed from Shin2 and BL1, *Pib* from BL1 and IRAT13, *Piz* from Fukunishiki, *Pi9* from WHD-1S-75-1-127, *Pi5*(t) from Moroberekan, *Pi12* from Moroberekan, *Pia* from Aichiashahi, *Pik-h* from K3, *Pik-s* from Shin2 and Caloro, *Pita* from K1, C105TTP2L9, C101PKT, Zhaiyeqing 8, and Yashiromochi, and *Pita-2* from Pi No.4, Reiho, and Fujisaka 5.

3.3 Genetic Characterization Using DNA Markers

These differential varieties were developed by backcrossing to their recurrent parents six times, and the degree of restoration in each differential variety varied depending on the number of backcrosses and recurrent parents (Fig. 2). Several introgression segments, which corresponded to the locations of blast resistance genes, were confirmed in almost all MLs and NILs with the genetic backgrounds of LTH, CO39, and US-2. A total of 28 lines among 31 MLs, 23 among LTH NILs, 14 among CO39 NILs, and 7 among US-2 NILs, contained detectable introgressions. As an example, the graphical genotypes on chromosome 11 are shown for the *Pik* allelic genes, *Pik, Pik-h, Pik-m, Pik-p, Pik-s*, and *Pi1*, and *Pi7*(t) in 30 lines (Fig. 3).

MLs: The genotyping for 31 MLs was carried out using 160 SSR markers distributed throughout the rice genome. Most of the MLs were developed by backcross breeding once or twice, while some lines were developed by backcrossing three to five times to the recurrent parent, LTH. The result demonstrated that the substitution to LTH ranged from 50 to 90.0% with an average of 77.3% among 31 monogenic lines. Three MLs, IRBLz-Fu, IRBLa-C, and IRBLk-Ka, exhibited frequencies of recurrent type markers of 50.0%, 58.1%, 52.5%, respectively, substantially lower than the expected level for single backcross breeding of 75% (Fig. 2).

LTH NILs:: The MLs have been further backcrossed with LTH to generate NILs. A total of 23 lines for 14 resistance genes, *Pia, Pib, Pik, Pik-h, Pik-s, Pita, Pita-2,*

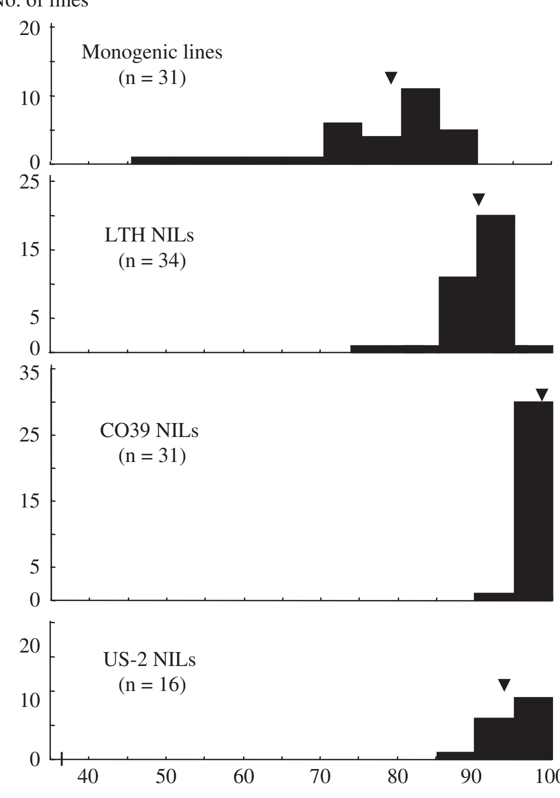

Fig. 2 Distributions of differential varieties for substitution ratio of recurrent parents' genetic backgrounds in each differential variety set. Triangle indicates the average among the differential varieties with same genetic background

Piz-5, Piz-t, Pi1, Pi3, Pi5(t), *Pi7*(t), and *Pi9* have been developed. All NILs were developed by backcrossing six times with the LTH as a recurrent parent. Polymorphism data of 160 markers revealed that the rate of genome restoration to the LTH parent ranged from 75.6 to 96.9% with an average of 90.6%. This range of genome restoration rate was lower than the expected level (Fig. 2).

CO39 NILs: A total of 31 CO39 NILs with at least to BC_6F_{10} generation have been developed for 14 resistance genes, *Pib, Pik, Pik-h, Pik-m, Pik-p, Pik-s, Pish, Pita, Pita-2, Piz-5, Piz-t, Pi1, Pi5*(t), and *Pi7*(t). This is the first differential variety set with an Indica-type genetic background for a large number of resistance genes. These NILs were analyzed using 161 SSR markers which were distributed in all 12 rice chromosomes. Results from the DNA analysis revealed that genetic components of the 31 NILs and all of the CO39 NILs showed that the rates of genome restoration to the recurrent parent exceeded 90%, with an average of 97.3% (Fig. 2).

US-2 NILs: LTH and CO39 provided good validations of susceptibility and have genetic backgrounds comparable to the differential varieties. However, the seeds of these two lines are difficult to obtain in sufficient amounts because they dislodge easily after heading under tropical conditions. US-2 was developed as a 'universal

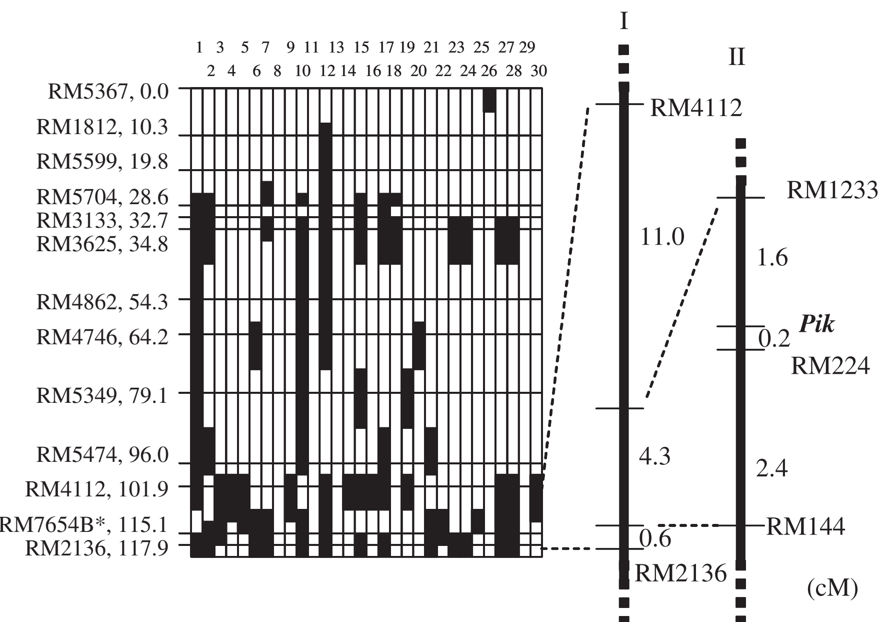

Fig. 3 Graphical genotypes of chromosome 11 of differential varieties introduced *Pik* alleles and *Pi7(t)*
Black regions on chromosome indicate the introgression from donor varieties.
* Tight linked DNA marker with resistance gene targeted.
I: Map by McCouch et al. (2002), II: Map by Fjellstrom et al. (2004).
1: IRBLk-Ka, 2: IRBLk-Ka[LT], 3: IRBLk-Ka[CO], 4: IRBLk-Ka[US], 5: IRBLk-Ku[CO], 6: IRBLkh-K3, 7: IRBLkh-K3[LT], 8: IRBLkh-K3[CO], 9: IRBLkh-K3[US], 10: IRBLkm-Ts, 11: IRBLkm-Ts[CO], 12: IRBLkp-K60, 13: IRBLkp-K60[CO], 14: IRBLkp-K60[US], 15: IRBLks-F5, 16: IRBLks-F5[US], 17: IRBLks-S, 18: IRBLks-S[LT], 19: IRBLks-S[US], 20: IRBLks-B40[LT], 21: IRBLks-Zh[LT], 22: IRBLks-CA[CO], 23: IRBL1-CL, 24: IRBL1-CL[LT], 25: IRBL1-CL[CO], 26: IRBL1-CL[US], 27: IRBL7-M, 28: IRBL7-M[LT], 29: IRBL7-M[CO], 30: IRBL7-M[US]

susceptible' rice line, which can be cultivated easily under any conditions in either tropical or temperate regions. A total of 16 NILs in US-2 genetic background have been developed for 12 rice blast resistance genes, *Pia, Pik, Pik-h, Pik-p, Pik-s, Pita, Piz-t, Pi1, Pi5*(t), *Pi7*(t), *Pi9*, and *Pi12*(t). Results from DNA analyses using 162 SSR markers revealed that chromosomal components of all US-2 NIL were almost identical to those of the US-2 parent. All US-2 NILs were developed by backcrossing six times except for IRBLks-F5[US]. The genome restoration rate for US-2 NILs ranged from 88.9 to 98.8% with an average of 94.6% (Fig. 2).

4 Discussion

Monogenic lines were used in the first set of international differential varieties which targeted a large number of resistance genes while harboring only a single one in

each genetic background (Tsunematsu et al., 2000; Kobayashi et al., 2007). These differential varieties are useful tools to clarify the pathogenicity of blast races and to identify the resistance genes in rice varieties based on the gene-for-gene theory (Flor, 1971). These varieties can provide useful information for breeding programs to improve blast resistance and are also useful materials for genetic and molecular biological studies into blast resistance mechanisms. IRRI and JIRCAS have continued to work on the development of blast-resistant NILs, after releasing the monogenic lines. A set of NILs is the most suitable material for race differentiation. NILs with LTH and CO39 backgrounds can be used as gene sources in breeding programs for Japonica and Indica-type rice varieties, respectively. Although NILs with a US-2 genetic background will be a more useful tool, they are still under development and may take some time to get pure lines.

Although these differential varieties are useful for rice breeding, the process to develop such materials is both laborious and time-consuming. The varieties were developed by backcross breeding and the existence of the resistance genes in the backcrossed lines was investigated by inoculation with a suitable blast isolate in every generation. Selection of differentials is very difficult when the resistance gene introduced from the donor parent harbors more than one resistance gene. Several differential varieties, 14 in MLs, 11 in LTH NILs, 17 in CO39 NILs and 9 in US-2 NILs were expected to contain some minor or other major resistance gene(s) that were additionally introduced in these genetic backgrounds. The donor varieties, Shin2, BL1, IRAT13, Fukunishiki, WHD-1S-75-1-127, Moroberekan, Aichiashahi, K3, Caloro, K1, Zhaiyeqing, and Yashiromochi, Pi No.4, Reiho, and Fujisaka 5 were used as other sources for resistance genes. These results demonstrate the difficulties of introducing resistance genes from varieties that harbor multiple resistance genes in their genetic background, based on the inoculation test with a blast isolate. Moreover, we were able to demonstrate the linkage between *Piz-t* from two donor varieties, Toride 1 and IR56, and the photosensitive gene, *Se-I*, in the middle of chromosome 6 in four differential varieties, ML, LTH NILs, CO39 NILs and US-2 NILs. These results made it clear that selection by the inoculation test using avirulent blast isolates is not an adequate method to remove the additional trait that is tightly linked with the resistance gene. However, this information will be useful in gaining an understanding of the relationship between resistance genes and the other agronomic traits.

The analysis at the molecular level using DNA markers will help in the confirmation of the existence of single resistance genes. The availability of various DNA markers in the public domain as well as information on the location of resistance genes in the rice chromosome could prove to be very useful information for determining the introgression of the resistance gene in the isogenic lines. We have analyzed the DNA of four sets of differential varieties and examined the chromosomal repeat distribution of each line through graphical genotype analysis using approximately 160 selected SSR markers (McCouch et al., 2002). The results revealed that the chromosomal repeat distribution of differential varieties of NILs is more uniform than those of MLs compared to the respective recurrent parent. For instance, the genome restoration rate of the recurrent parent of 31 MLs with

LTH genetic background varied from 50 to 90% with an average of 77.3%, while 34 NILs which were developed by backcrossing six times using the same genetic background, LTH, showed genome restoration rates from 75.6 to 96.9% with an average of 90.6%, which was lower than the expected rate of 99%. Based on the polymorphism data of SSR markers obtained in this study, it was shown that the average genome restoration rate of CO39 NILs with Indica-type variety genetic background and US-2 NILs with the hybrid Indica-Japonica genetic background was 97.5 and 94.6%, respectively, higher than the average of LTH NILs, 90.6%. The degree of genome restoration varied according to the number of backcrosses or type of recurrent parents among universal differential variety sets and these corresponded with the phenotypic variations (data not shown). We have identified several introgression segments on the genome of differential varieties corresponding to the location of blast resistance genes, which had been previously reported. Therefore, these lines require further study to clarify the introgression of the resistance genes. This can be done through segregation analysis between DNA markers and resistance genes. The information on DNA markers in each differential variety will be useful in blast resistance breeding for targeting these genes and removing the additional chromosome segments in these genetic backgrounds. Furthermore, the association between undesirable introgression segments, which were detected in some differential varieties with the resistance genes, should be clarified.

In addition to the 24 targeted genes, lines for other types of resistance genes including QTL, field resistance and panicle blast resistance will be developed with these genetic backgrounds, to clarify the effect of each gene and QTL and their applications in more advanced studies such as gene pyramiding.

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Sequential Planting as a Method of Screening of Durable Resistance to Rice Blast in Korea

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Abstract A sequential planting method was developed to identify durable blast resistance of rice cultivars against a mixture of 26 rice blast isolates. Daesanbyeo and Gihobyeo which, initially showed high resistance in farmer's field followed by rapid breakdown of resistance in a short period, exhibited low disease until the third and fourth plantings but later showed higher than 40% diseased leaf area (DLA). In contrast, Palgongbyeo and Seomjinbyeo with low disease occurrence and sustainable field resistance during the last 20 years in the farmers' field, showed less than 20% DLA until the seventh planting. Results from the sequential planting method in the greenhouse were significantly similar to the farmers' field data, suggesting that the method is effective to evaluate durability of rice blast resistance. Durability of 22 rice cultivars/lines was also examined. Monogenic rice lines, IRBL5-M(*Pi5(t)*) and IRBL3-CP4(*Pi3(t)*) exhibited durable resistance to Korean rice blast isolates below 40% DLA during the sequential planting times. Interestingly, susceptible symptoms emerged from IRBL9-W(*Pi9(t)*), known to be associated with broad-spectrum resistance, since the third planting time, and disease severity of IRBL9-W extremely increased from the fourth planting time. Suwon474, Suwon476, and Gyehwa22 were identified as durable resistant cultivars to Korean blast isolates by the sequential planting method. This work will be applied to establish the new Korean differential system for blast resistance breeding program.

Keywords Durable resistance · Rice blast · Sequential planting

1 Introduction

Rice blast is pathogenic to almost 40 plant species in 30 genera of the *Poaceae* including *Oryza* (Asuyama 1965). Rice seedlings and plants at the tillering stage are often completely killed under conditions favorable to the pathogen. The pathogen

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attacks leaf, culm node, and panicle neck node. Infection of the panicle base, called neck blast or rotten neck blast, is the most destructive symptom in most environments where blast is a problem (Asuyama 1965). Heavy infections of the panicle are often detrimental to rice yields. Yield losses due to blast have been reported to be more than 80% in extreme cases (Exconde and Raymundo 1970; Padmanabhan 1965). Average yield losses have been reported to be approximately 3–4% in Japan (Goto and Yamanaka 1968; Ou 1985). In Korea, yield loss due to the blast disease was reduced from 6% in 1960 to 0.02% in 1990, however, the amount of chemical control was increased by five times during that same period (Kim 1994). The most important methods for blast control are the cultivation of resistant varieties, fungicide application, and cultural practices. The use of resistant cultivars is an inexpensive, effective, and environmentally sounds approach to control blast disease (Ahn and Seshu 1991). However, resistance is generally short-lived, when conferred by a single gene or a few major genes (Ahn and Seshu 1991; Bonman et al. 1992). Resistance by major genes is highly race specific and expressed qualitatively (Bonman et al. 1989). In the last several decades, researchers have been striving to breed for resistance against blast disease and many resistant cultivars were developed. However, resistance of these cultivars broke down within a few years after their commercial use (Han 1995; Kiyosawa 1982). The abrupt breakdown of major gene resistance could be due to the build up of a pre-existing subpopulation or the rapid adaptation of the fungus when subjected to the selective pressure exerted by resistant cultivars. Hence, quantitative (race non-specific) resistance becomes more important than qualitative resistance in breeding strategies. Durable resistance is defined as "Resistance that remains effective when planted for a long time over a large area" (Johnson 1984). Since quantitative resistance is controlled by multiple genes with small effects, it is considered to be more durable. In addition, individual resistance genes may contribute in breeding for durable resistance.

Current resistance screening methods are more applicable to qualitative resistance detection and are not suitable for evaluation of durable resistance. Here we describe the development of a durable resistance screening system called the sequential planting method. To provide useful information in breeding programs, we also evaluated the durability of some monogenic lines and elite-lines in Korea.

2 Epidemic of Rice Blast Disease and Breakdown of Resistance in Korea

Korea has thousands of years of history of rice cultivation (RDA 1990). There are several historical records of rice blast epidemics in Korea (Lee 1979). In most cases there are two factors associated with the epidemics; one is climatic conditions favorable to blast, and the second is the genetic vulnerability of commercial cultivars either from incomplete resistance or from the build up of new pathogenic races. Severe economic losses of rice due to the blast disease have been reported in many countries. Blast epidemics in Japan resulted in approximately 700,000 tons of

yield loss in 1953 (Ou 1985). Although fungicides were used extensively to control blast, the mean yield loss by blast disease approached 3% during 1953–1960 in Japan (Goto et al. 1961). There have been many reports of outbreaks of rice blast in India, the Philippines, South America, and Africa (Ou 1985). In Korea, outbreaks of panicle blast were reported in 1917, 1926, 1928 and 1936. In 1928, severe blast incidences were investigated all over the country and yield loss was up to 18% in 1929. The average yield loss of rice due to blast during the ten years from 1964 to 1973 was reported to be 4% in Korea (Lee et al. 1975). There was a drastic epidemic of rice blast in 1978, when incidence of panicle blast was more than 40% in some cultivars. Lee et al. (1975) investigated the outbreak of blast in 1978 and concluded that the outbreak resulted from a combination of susceptibility of commercial cultivars and the wide distribution of pathogenic races, along with favorable climatic conditions, such as frequent rainfall, high humidity, decreased sunshine and less solar radiation.

Most of the Korean commercial cultivars planted during the early 1960s were regarded to have the resistance gene, *Pi-a*, for blast (Lee et al. 1975). Since severe incidence of blast had been frequently observed, a new resistant variety Kwanok possessing R gene *Pi-k* from Kanto55 was released into farmers' field in 1966. However, the variety was unfortunately severely infected in 1969 by a new race at the panicle stage (Lee 1979).

A semi-dwarf gene from *Indica* rice, TN1, was introduced into *Japonica* rice Yukara in 1965 (RDA 1990). Since then, *Indica* rices have been extensively used to make *Tongil-type* varieties by crossing with *Japonica* lines (RDA 1990). The successful performance of *Tongil-type* varieties during the years from 1971 to 1977 was responsible for the so called "Green revolution in Korea." The yield of rice increased from 3.67 MT/ha in 1972 to 5.37 MT/ha in 1977 (Lee 1978). Although the main objectives for the introduction of *Indica* genes into *Japonica* rices were to increase yield capacity by shortening culm length to reduce lodging, erect plant types to enhance the photosynthesis, and tolerance to the high fertilizer levels, resistance to blast was one of the important outcomes of the *Tongil-type* rices (RDA 1990).

There have been no severe blast epidemics during the seven years since *Tongil-type* varieties were released to farmers. However, panicle blast occurred regionally on rice cultivars, Tongil and Yushin in Jeonbug province in 1976 (Ryu et al. 1987). Severe blast epidemics were observed in all provinces in 1977, and in 1978, 57.4% and 40.7% of panicles on average were infected on rice two cultivars, Raegyeong and Nopung, respectively. Although the outbreak of blast in 1978 was partially from favorable environmental conditions, it was a typical case of the breakdown of resistance of rice to blast in Korea (Yamanaka and Kang 1980). Breakdown of resistance of *Tongil-type* varieties has been verified by analysis of pathogenic diversity and population structure for that year (Ryu et al. 1987). Resistant cultivars, such as Youngpungbyeo and Gayabyeo, became susceptible to the new pathogenic races KI-315a and KI-315b. Race KI-401 was a newly identified race, which can infect resistant cultivars Seonambyeo and Cheonmabyeo. There have been no resistant cultivars showing overall resistance to all the races distributed in the field since 1983. Race KI-409 was first identified from Namyangbyeo in 1985 and has rapidly

built up since 1990. Most commercial cultivars having BL1 and BL7 pedigrees were susceptible to race KI-409. Race KI-409 was isolated from 47 rice varieties and became predominant with 23.7% of distribution ratio in 1995.

3 Selection of Representative Pathogen Races in Korea

Selection of representative pathogen races, which can represent each pathogen population, is required for screening of durable resistance. Conventionally, differential systems for characterizing the rice blast fungus have been developed and pathogen populations of *P. grisea* have been characterized in time and space in many rice growing countries (Ou 1985). However, the procedure for pathotype identification and the interpretation of the virulence bioassay are often ambiguous or inconsistent, which could result in contradictions in pathotype designation. Furthermore, pathotype identification is dependent upon differential cultivars chosen from limited resources of both the host and the pathogen, which may not sufficiently reflect the pathotypes in nature. Hence the pathotype identification may not well characterize the genetic structure of the pathogen population.

As an alternative and/or complement to the conventional virulence typing, the direct characterization of genomic DNA of the fungus is now possible by restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) analyses (Valent 1990; Leung et al. 1993). For the blast pathogen, dispersed repeated DNA sequences called MGR has been developed as a hybridization probe for genome RFLP analysis (Hamer 1991). A good relationship between lineages defined by MGR586 and pathotypes of rice infecting isolates of *P. grisea* was reported by Levy et al. (1991), but complex relationships between genetic lineages and pathotypes were detected in the isolates from Philippines and Colombia (Correa et al. 1993; Zeigler et al. 1995). Xia et al. (1993) found a promising possibility between pathotypes and genetic lineages generated by MGR probes. Genome analysis is now the most extensively used technique for analyzing the phylogeny and the structure of within and between pathogen populations. However, Korean isolates have been showed the low relationship between the pathotypes and MGR-RFLP types (Han et al. 1996). In this study, we selected representative Korean isolates for use in a three-step screening method for durable resistance. First, pathotypes of thousands of isolates collected from farmer's fields since 1980 were classified using different Korean varieties. Second, selected representative pathotypes were reclassified using molecular marker MGR586. Third, the *M. grisea* isolates were screened with monogenic resistant lines. Finally, we selected 26 Korean isolates, which represent Korean *M. grisea* population.

4 Development of Sequential Planting Method for Screening of Durable Resistance to Rice Blast Disease

Generally, three kinds of resistance screening methods have been used in blast resistance breeding programs. The methods are artificial inoculation of rice seedling in the greenhouse, nursery screening (hot spot) in many different locations, and field

tests. These methods are very effective in screening of qualitative resistance but they are hard and the examination of the durability of rice cultivars takes a very long time. Since these methods have chiefly targeted genes displaying major effects, it has been observed that duration of lasting effect of resistance was either extremely short or not significant from time to time. Until now, there are no reports concerning the evaluation of the durability of resistance to blast disease in rice. Hence we conducted the sequential planting method, which allows for the screening of durable resistance under artificial conditions in a short period of time. This research was carried out to evaluate the effectiveness of the new method (sequential planting method) in detecting durable resistance and in judging the durability of monogenic resistant lines and some Korean elite lines.

4.1 Materials and Methods

In this study, we used six cultivars that are grown widely in farmers' field in Korea (Table 2). Twenty-six representative *M. grisea* isolates that including 23 Korean races were selected using the method as described above.

4.1.1 Sequential Planting

The six rice cultivars were directly seeded on soil and covered with bed soil in the rectangular plastic container ($40 \times 30 \times 10$ cm). A spore suspension (1×10^5 spore/ml) was sprayed on the 4–5 leaf grown plants from the first seeding. Then the plant infection was induced naturally from the 2nd to the 7th seedings planted at two week intervals. We named this method the "Sequential Planting Method." For sufficient infection, we put a healthy plants pot closely next to the infected plants for 3–4 days. During the infection inducing period, we covered the plants with transparent plastic film at night to keep high humidity (Fig. 1).

4.1.2 Evaluation of the Disease Severity

For resistance evaluation, diseased leaf area (DLA) was measured seven days after inoculation using the standard evaluation system for rice (IRRI 1996). Conventionally, 40% DLA is used as the basis to indicate resistant severity in seedling screening method, thus in this study, 40% DLA was used as the criterion to determine the durability of resistance. The level of the durability of resistance was classified using a range of 0 to 7 using 40% DLA as standard point (Table 1).

4.2 Detection of Durable Resistance

Palgongbyeo and Seomjinbyeo have been cultivated for more than 20 years in farmers' field over a large area and are still showing resistance. Resistance to specific *M. grisea* races of Daesanbyeo and Gihobyeo broke down after within

Table 1 Level of durability of resistance based on temporal disease progress

Level of Durability	7	6	5	4	3	2	1	0
Description of disease	Planting time with over 40% DLA							Below 40% DLA
	from 1st	from 2nd	from 3rd	from 4th	from 5th	from 6th	at 7th	until 7th planting

three years of their release. On the other hand, Ilpumbyeo and Jinmibyeo exhibited severe susceptible reactions to race KI-409 just after they were released in farmers' field.

Durability of resistance was clearly divided between the cultivars based on DLA and sequential infection rate. Jinmibyeo and Ilpumbyeo showed DLA of 58% and 41%, respectively at the 1st planting where 1/3 of leaves were dried up by pathogenic lesions (Table 2). DLA of Gihobyeo and Daesanbyeo increased rapidly at the 3rd and 4th planting stages with over 60%. Palgongbyeo and Seomjinbyeo exhibited resistance with small number of susceptible lesions (below 20% DLA) during all the sequential planting times.

Using 40% DLA as standard point, durability of Palgongbyeo and Seomjinbyeo were rated 0 indicating these cultivars are conferred with high durability against *M. grisea*. Daesanbyeo and Gihobyeo displayed medium level of resistance marking durability index 3 and 4. Durability levels of Jinmibyeo and Ilpumbyeo were rated as 7 suggesting that these two cultivars do not have any durability at all.

The results revealed that the sequential planting method may well reflect durability of resistance, and also the results were consistent with actual result in farmers' field. In the case of Gihobyeo and Daesanbyeo, which can be rated as resistant based on one time inoculation, the sequential planting method was able to detect the change of disease incidence in the course of time. We believe the sequential planting method is an accurate and rapid way to evaluate durable resistance.

Table 2 Disease severity and durability of six rice cultivars by sequential planting method

Planting time	Diseased leaf area (DLA, %)					
	Palgongbyeo	Seomjinbyeo	Daesanbyeo	Gihobyeo	Jinmibyeo	Ilpumbyeo
1st	12.0	15.0	25.0	15.0	57.5	40.5
2nd	12.0	15.0	35.0	15.0	70.0	40.5
3rd	11.5	17.5	70.0	30.0	70.5	82.5
4th	11.0	5.0	60.0	60.0	60.0	65.0
5th	10.0	10.0	55.0	60.0	65.0	65.0
6th	5.0	20.0	85.0	85.0	87.5	90.0
7th	5.0	11.5	95.0	85.0	95.0	90.0
Level of durability	0	0	5	4	7	7

5 Durability of Monogenic and Korean Elite Lines

There are several ways to increase the durability of rice cultivars against pathogens such as accumulation of minor genes, pyramiding of major genes, and breeding a combination of minor and major genes. To achieve durable resistance, the selection of useful candidate genes is required prior to actual breeding.

Durability of 22 rice cultivars/lines has been examined by the sequential planting method. The monogenic line with *Pik-s* showed 40–62% diseased leaf area (DLA), and *Pib, Pit*, and *Pita-2* exhibited 70–98% DLA after the second planting. *Pita, Pi19*(t), and Suwon477 were highly susceptible to Korean *M. grisea* isolates with over 80% DLA during all times of the sequential planting (Table 3, Fig. 2A). Ilpumbyeo, Iri401, and *Pi* showed low DLA of 27–32% at the first planting time, but the DLA increased rapidly to 65–82% from the second planting time (Fig. 2B).

Monogenic lines, IRBL5-M(*Pi5(t)*) and IRBL3-CP4(*Pi3(t)*) exhibited durable resistance to Korean rice blast isolates with DLA below 40% during the sequential planting times. Suwon474, Suwon476, and Gyehwa2 were identified as durable resistant lines to Korean blast isolates by the sequential planting method along with the durable resistant control cultivar Palgongbyeo (Fig. 2B). Interestingly, susceptible symptoms emerged from IRBL9-W *(Pi9)*, a line known to be associated with broad-spectrum resistance. Disease severity of IRBL9-W had increased in the third

Table 3 Disease severity and durability of 22 elite breeding lines and monogenic lines in seven sequential plantings

Cultivar	Disease leaf area (%)							Level of durability
	1st	2nd	3rd	4th	5th	6th	7th	
IRBL9-W	4.0	3.5	17.5	57.5	57.5	52.5	50.0	4
IRBL5-M	8.0	4.0	14.0	25.0	25.0	30.0	32.5	0
IRBLta-K1	87.5	98.0	98.0	98.5	98.0	98.5	91.5	7
IRBLzt-T	17.5	24.0	55.0	74.5	60.0	37.5	52.5	5
IRBLi-F5	27.5	65.0	81.5	84.5	87.0	98.5	84.0	6
IRBL3-CP4	15.0	15.0	22.5	25.0	20.0	22.5	27.5	0
IRBLb-B	80.0	94.0	98.0	98.5	98.0	98.5	98.0	7
IRBLks-F5	40.0	55.0	55.0	62.5	55.0	57.5	62.5	7
IRBL19-A	87.5	95.0	92.5	97.0	87.0	85.0	75.0	7
IRBLt-K59	50.0	55.0	52.5	67.5	55.0	57.5	55.0	7
IRBLta2-Re	42.5	70.0	90.0	98.5	59.5	50.0	67.5	7
Suwon478	6.5	26.5	10.0	12.2	11.0	10.0	17.5	0
Suwon474	22.5	27.5	30.0	35.0	30.0	25.0	20.0	0
Cheolwon63	3.0	1.5	1.5	3.0	8.0	20.0	15.0	0
Iksan458	25.0	30.0	32.5	50.0	45.0	32.5	37.5	4
Iksan456	20.0	30.0	37.5	50.0	45.0	25.0	55.0	4
Gyehwa22	25.0	25.0	35.0	30.0	27.0	22.5	30.0	0
Iri401	32.5	77.5	97.0	97.0	97.0	75.0	79.0	6
Palgongbyeo	15.0	15.0	27.5	35.0	25.0	15.0	20.0	0
Ilpumbyeo	32.5	82.5	97.0	98.5	70.0	80.0	84.0	6
Suwon476	15.0	17.5	14.0	14.0	13.0	11.0	15.0	0
Suwon477	80.0	90.0	94.0	97.0	88.0	75.0	82.5	7

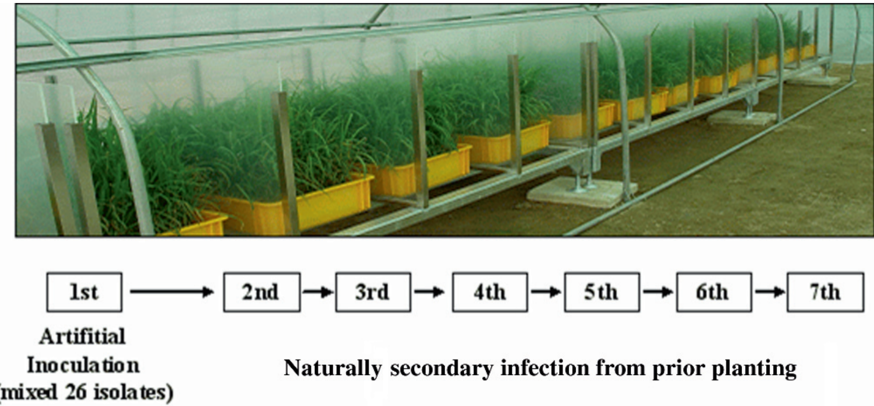

Fig. 1 Sequential planting method for identification of durability to rice blast disease (Kim et al. 2004)

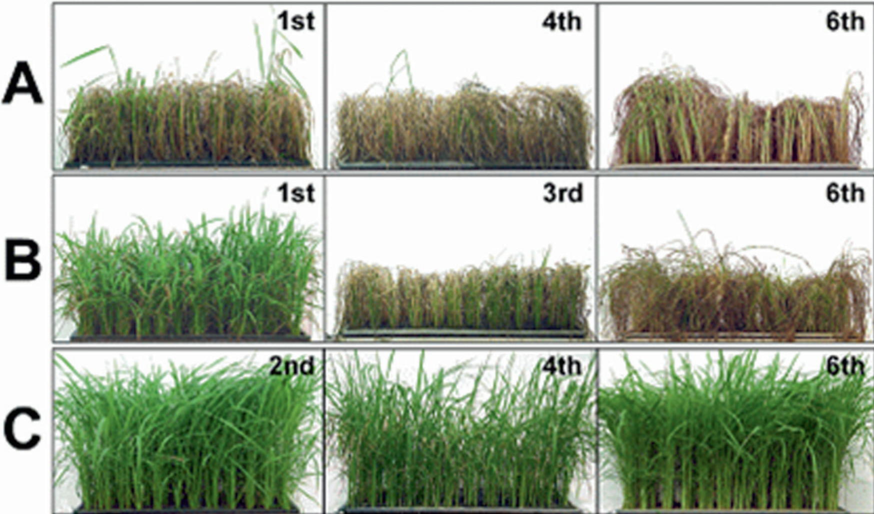

Fig. 2 Blast disease development of rice cultivars: (**A**) IRBLta-K showing susceptible reactions in all times of sequential planting; (**B**) Ilpumbyeo showing susceptible reactions beginning at the 2nd planting time; and (**C**) Suwon476 showing durable resistance during the sequential planting

planting and was extremely severe in the fourth planting, suggesting that *Pi9 (t)* will not be useful for durable resistance in Korea.

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Development of a Package of 20 Varieties for Blast Management on Upland Rice

Suwarno, Erwina Lubis, Aris Hairmansis and Santoso

Abstract Blast disease caused by *Magnaporthe oryzae* is the most important constraint to high yield of upland rice in Indonesia. The pathogen has diverse and dynamic races resulted in its capability to rapidly break down of the improved resistant varieties. Many improved varieties with blast resistance have been released in Indonesia, however, the resistance was broken down after three consecutive planting seasons. Genetic diversity of blast resistance could be the important factor that should be consider for durable field resistance and blast management. A package of 20 varieties of upland rice with diverse blast resistance has been developed. The varieties of the package were selected from advanced breeding lines developed in the national breeding program with the criteria of agronomical characters, grain quality, and diverse blast resistance. Selection for agronomic characters was conducted in Tamanbogo Research Station located in upland area with the criteria of better phenotypic acceptability and higher yield than the traditional check varieties. Selection for grain quality was done based on the grain quality data obtained from the Grain Quality Laboratory of Indonesian Center for Rice Research (ICRR). Selection for diverse blast resistance was conducted based on the diversity of the reaction patterns to 19 pathogen races artificially inoculated in the phytopathology laboratory. Based on the selection criteria, 20 lines were selected out of 500 with the following characteristics, diverse blast resistance, 75– 40 cm plant height, 104–120 days maturity, 7–14 tillers per hill, slender-medium grain shape, and 18.1–28.1% amylose content. The selected lines and some lines selected by farmers are now being evaluated at four locations of upland areas where 20 lines will be selected for the package.

Keywords Upland rice · Durable resistance · Field selection · Agronomic traits

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1 Introduction

Annual cultivated area of upland rice in Indonesia was almost constant at about 1.2 million ha. The upland rice contributes a small portion to the national rice growing area and production which were about 9 and 5%, respectively. Despite the small contributions, upland rice is the major source of food and income for millions people living in the upland areas. Increasing the upland rice production, therefore will improve food security and income of the people.

Indonesia has 35.5 million ha upland area potential for agricultural development (Hidayat et al., 2000), among which 5.1 million ha are potential for food crop cultivations including upland rice (AARD Research Team, 1998). The figure of the potential land is much higher than 1.2 million ha of the existing cultivated area of upland rice in the country. In the upland areas, rice is cultivated traditionally where traditional varieties are grown without or with low rate of fertilizers. The national averaged yield of upland rice in Indonesia was 2.58 t/ha (BPS, 2005), while high yields of 3.5–6.6 t/ha could be obtained by applying improved cultivation practices (Toha, 2000). There is a great potential to increase rice production in uplands through expansion of the growing area as well as improvement of cultivation practices to increase the yield.

One of the major constrain to high yield of upland rice is blast disease caused by *M. oryzae*. The pathogen attacks plant leaf during the vegetative stage as well as panicle during the generative stage causing leaf and panicle blast, respectively. The pathogen has diverse and dynamic races enabling it to rapidly break down improved varietal resistance. Improved rice varieties with blast resistance became susceptible to the disease after consecutive cultivation for 2–3 planting seasons (Amir and Nasution, 1995). In spite of many improved varieties with blast resistance that have been released, the farmers in upland areas still cultivate traditional varieties that have stable blast resistance and perform stable yield even though it is low.

Many efforts to improved varietal resistance to blast have been conducted including inheritance study (Kustianto et al., 1995), identification of resistant genes (Amir and Nasution, 2001), development of molecular markers (Bustamam et al., 2001), and breeding for blast resistance (Suwarno et al., 2001). It was indicated that genetic diversity was the major factor contributing to durable blast resistance which should be considered for the disease management. In order to improve the yielding ability and genetic diversity, a package of 20 varieties with diverse blast resistance is being developed.

2 Genetic Diversity of Blast Pathogen

Blast patogen has diverse and dinamic races, it also has capacity to develope new races. Using the national defferential varieties consisting of seven rice varieties, more than 30 different races were identified from the pathogen isolates collected from uplad rice areas in Indonesia (Amir et al., 2003). Molecular analysis also

indicated the diversity of the pathogen population. Based on the *MGR 586* marker, 16 different haplotypes were identified (ARBN, 1997), while using specific marker of *Pwl2, Erg2* and *Cut1*, 8 different haplotypes were found (Reflinur, 2005).

Monitoring on blast pathogen population conducted in upland rice areas in Lampung during 2000–2004 revealed that there were at least 13–17 pathogen races presented in each planting season (Table 1). A total of 26 pathogen races were detected during the five years monitoring among of which seven races, 001, 023, 033, 073, 101, 133, and 173 appeared in all of the years. On the other hand, there were 10 races appeared only once during the period of the monitoring.

Amir et al. (2003) also reported that during the wet season 2003, a total of 30 races were identified in rice production areas of West Java Province. Six out of them were the new races detected in West Java i.e. races 301, 333, 341, 343, 353, 361, 371 and 373. These new races known to be more virulent than the races found before. Several races were also found in the irrigated lowland while blast incidence was increasing in this area, especially neck blast disease.

Table 1 Race composition changes of blast pathogen isolated from upland rice cultivation in Lampung during 2000–2004

		Composition (no of isolates in %) in year			
No.	Pathogen race	2000	2001	2003	2004
1	001	3.37	2.68	2.58	5.71
2	003	–	1.33	0.64	8.57
3	011	–	1.33	–	–
4	013	0.83	2.68	0.64	–
5	021	–	–	0.64	–
6	023	3.53	1.33	7.09	2.85
7	033	17.86	20.16	20.00	51.42
8	041	–	5.37	–	4.28
9	043	–	–	–	2.85
10	053	–	10.75	–	1.42
11	061	0.83	2.68	–	–
12	063	–	–	0.64	–
13	071	–	1.33	–	–
14	073	1.7	5.37	1.29	5.71
15	101	2.08	1.33	1.29	1.42
16	103	–	1.33	–	–
17	123	1.86	–	1.29	1.42
18	133	13.72	2.68	36.77	5.71
19	141	0.83	–	0.64	–
20	143	–	2.68	–	1.42
21	161	0.83	–	–	–
22	163	3.33	1.33	–	–
23	171	1.03	–	–	–
24	173	48.06	35.53	18.06	7.14
25	333	–	–	2.58	–
26	373	–	–	5.80	–
No.of races		14	17	15	13

Table 2 Improved upland rice varieties released in Indonesia since 1994

Varieties	Year released	Maturity (days)	Cooked rice texture	Yield (t/ha)	Important characteristics*
Gajah Mungkur	1994	95	Medium	2,5–3,5	Bl, Dr
Way Rarem	1994	105	Hard	3,0–4,0	Bl, A1, Fe
Kalimutu	1994	95	Medium	2,5–3,5	Bl, Dr
Jatiluhur	1994	115	Hard	2,5–4,5	Bl, Sh
Cirata	1996	120	Medium	3,0–5,0	Bl, BPH 1, Sh
Limboto	1999	105	Medium	3,0–5,0	Bl, A1, Dr
Towuti	1999	120	Soft	3,0–5,0	Bl, BPH 2, 3, BLB
Batutugi	2001	116	Soft	3,0–5,0	Bl, Al, Dr
Danau Gaung	2001	113	Medium	3,4–5,0	Bl, BS, Al, Fe
Silugonggo	2001	90	Medium	4,5–5,5	Bl, HDB
Batang Gadis	2002	122	Medium	3,6–5,6	Bl, Aromatic
Situ Bagendit	2002	105	Soft	3,0–5,0	Bl, BLB
S. Patenggang	2002	115	Soft	3,0–5,0	Bl, Aromatic

* Bl, BPH, BLB, and BS: resistant to blast, brown planthoper, bacterial leaf blight, and brown spots, respectively; Dr, Al, Fe, and Sh: tolerant to drought, Al toxicity, Fe toxicity, and shading, respectively

3 Stability of Blast Resistance

Since blast is the major disease constraining high yield of rice in upland area, resistance to the disease is always put at the top priority in the national breeding program for upland rice. All of the improved high yielding varieties officially released in Indonesia were resistant to the pathogen (Table 2). It was demonstrated in farmers' fields that cultivation of the improved varieties with improved cultural practices including application of organic manure, proper rates of N, P, and K fertilizers, and weed management could produce high yield of 3.5–6.6 t/ha, while the neighbouring farmers' fields only yielded 2.5–3.0 t/ha (Toha, 2000). However, the adoption of the improved varieties has been very low mainly due to the rapid break down of the blast resistance.

There was a development program to increase upland rice production through introdution of improved varieties for intercroping under young tree crop plantations. Rice seed was distributed to farmers in the plantation areas of ruber and oil palm with the total area of about 100,000 ha during 1994/1995 cropping season. The rice crop performed well yielded 3–4 t/ha while the national yield average of upland rice was 2.5 t/ha (Directorate General of Plantation, 1995). The area of rice cultivation, therefore, was about doubled to 215,000 ha in the following planting season. However, the rice cutivation could not sustain mainly due to blast disease

Introduction of improved varieties should be followed by monitoring of blast pathogen population. Based on the reaction patterns of the differential varieties to the pathogen population, predominant races could be predicted and the resistant varietiy could be selected for the following planting season. The populations and their changes could be different among locations and so the suitable resistant varieties. Therefore, the method was not practical especially for the upland rice.

Table 3 Ranges of some characteristics and blast symptom of lines derived from the respective traditional varieties

No	Varieties	Plant height (cm)	No. of tillers	Maturity (days)	Blast symptom (%)
1	Dayang Rindu	121–150	5–15	118–120	94–100
2	Brentel	111–139	3–13	108–126	22–61
3	Sirendah	109–152	4–13	124–129	22–39
4	Sirendah PG	107–138	3–16	111–114	22–39
5	Sirendah Putih	98–144	3–14	113–119	94–100
6	Karang Umpu	101–142	2–12	137–143	33–61
7	Ketan Utri	121–160	2–9	122–128	33–100
8	Grendel	112–160	2–11	118–124	22–44
9	Ketan Serang	116–139	10–17	93–104	72–100
10	Selegreng	99–120	5–16	103–105	17–33
11	Lampung Lawer	125–135	5–15	136–144	28–100
12	Lampung Putih	107–138	4–17	113–119	39–72
13	Lampung Kuning	119–155	5–15	122–125	56–100
14	Lampung Arak	115–154	3–11	105–111	22–67
15	Ketan Hitam	102–132	6–23	103–111	89–100
16	Serampat	123–140	6–13	130–136	100
17	Klemas	125–135	5–10	136–140	28–50
18	Sagi	150–160	5–10	101–110	22–67

Generally, upland rice is still cultivated traditionally. Many different traditional varieties with low tillers, long stem, and low yield are cultivated in most uplands areas. Although low yield is obtained, the traditional cultivation has no risk of fail since it is never seriously damaged by blast disease. These indicated that traditional rice varieties had stable or durable blast resistance. Genetic diversity could be the major factor contributing to the stable blast resistance. Exploration conducted in an upland rice area in Central Lampung district could collect 18 traditional varieties. Two or more varieties each of which were not uniform, could be found in a farmer's fields of less than one ha area. Individual or panicle selection showed that the lines derived from the traditional varieties have significant morphological variability among lines within and inter varieties (Table 3). The variability among lines was also observed on blast resistance. The result of blast resistance evaluation conducted by artificial inoculation of two blast races to the lines (Table 3) indicated the diversity of the lines. These indicated that the traditional varieties were not pure varieties but mixture.

4 Development of Package of 20 Blast Resistant Varieties

Breeding programs on blast resistance in Indonesian Center for Rice Reseacrh (ICRR) have considered the genetic diversity by using different genetic sources. More than 30 blast resistant varieties of traditional and introduced have been crossed for the breeding programs. However, not all or even only few of the genetic variability were included in the released varieties since only the best or top yielding varieties could be officially released. There were some development programs to

increase upland rice production where introduction or deployment of the improved varieties was the major component, but only one or two newly released varieties were included. Those systems of varietal release and deployment of the improved varieties caused the low genetic diversity of the improved variety cultivated which could not meet those of the blast pathogen and the resistance was rapidly broken.

Selection for phenotype acceptability in the breeding program was done based on the line performance relative to the check varieties, the best released varieties. The selected advaced breeding lines from the observation nursery and beyonds, therefore, have phenotypic acceptability and yield better or similar to that of the improved varieties which are much higher then those of traditional varieties that are cultivated by most of the farmers in the uplands. Most of the lines were unused since only the best few of them passed the variety release procedure.

In order to increase the yield of the upland rice while the genetic diversity is maintained to avoid the blast problem, a package of 20 varieties with diverse blast resistance is being developed. Deployment of the package replacing the traditional varieties will develop mosaic cultivation of improved varieties with high yield and diverse genetic of blast resistance. The cultivation, therefore, will be more or less similar to the traditional ones but with improved varieties.

The important characteristics of the advaced breeding lines included in the package would be:

- Diverse blast resistance;
- High yield, higher than those of the traditional varieties;
- Tolerance or moderately tolerance to Al toxicity;
- Acceptable grain and cooked rice quality including: medium-long grain length, medium-slender grain shape, low chalkiness, and soft texture of cooked rice

The development of the package of 20 varieties has been conducted through selection of the desirable characteristics on the existing breeding materials of about 500 advaced breeding lines evaluated in the observation, preliminary yield trials and advaced yield trials. The status of the breeding materials was as follows:

- The lines were derived from 80 crosses including diverse sources of blast resistance;
- The lines have better agronomic characteristics and higher yield compared to those of traditional varieties since they were selected in the previous steps of the breeding program;
- Most of the lines have tolerance to Al toxicity since the major breeding site for upland rice is in acid soil where Al toxicity constraints high yield of susceptibe breeding lines.
- Some of the lines have also been selected for good and acceptable grain quality.

Based on the material status, selections for the package were foccused on the blast resistance diversity and desirable grain qualities. Selection for blast resistance was conducted in a greenhouse through artificial inoculation of 19 pathogen races available in the ICRR's collection. Lines with different paterns of resistance to the pathogen races were selected. It was expected that the lines carrying diverse

genes for blast resistance. Grain quality selection was done through laboratory analysis conducted for grain quality characters including grain length, grain shape, chalkiness, and amylose content which is closely related to the cooked rice texture.

Based on the selection criteria, 24 lines were selected for the development of the package. Besides, 6 promising blast resistant lines which were selected through Participatory Varietal Selection and cultivated by some farmers were also included, those are TB490C-TB-1-2-1, TB361B-30-6-2, BP1976B-2-3-7-TB-1-1, TB356B-TB-18-3, IR30176, and IR60080-23. The lines performed blast resistance in the field but not tested yet against the diverse pathogen races. The reactions of the selected lines were listed in Table 4.

The selected lines performed relatively good agronomic characteristics compared to the traditional varieties with ranges of 71–148 cm plant height, 7–14 tillers/hill,

Table 4 Reaction of selected lines to 19 blast pathogen races

No	Lines	Blast pathogen races									
		001	003	031	101	141	211	263	373	041	033
1	TB490C-TB-1-2-1-MR-4-29	R	R	MR	R	MR	S	R	S	R	R
2	TB490C-TB-1-2-1-MR-1-1	R	MR	MR	R	S	R	R	S	R	R
3	B11577E-MR-B-12-1	R	R	R	MR	S	MR	MR	MR	R	R
4	B11577E-MR-B-12-1-1	MR	MR	MR	MR	S	R	–	R	R	R
5	B11577E-MR-B-13-1-1-5-5	MR	MR	R	MR	MR	MR	–	R	R	MR
6	B11577E-MR-B-13-4	R	R	MR	R	S	MR	R	MR	R	MR
7	IR65907-116-1-B-MR-4	R	MR	MR	R	MR	R	MR	S	R	R
8	B11602E-TB-1-2	R	R	R	R	S	MR	R	S	MR	R
9	B11602E-TB-1-3	R	MR	MR	R	S	S	MR	S	R	R
10	B11602E-TB-2-4-3	R	MR	R	R	MR	R	–	S	R	MR
11	TB409B-TB-14-3	R	R	S	R	S	MR	MR	S	R	R
12	B11593-MR-11-B-2-8	R	R	MR	R	S	R	R	MR	R	MR
13	B10580E-KN-28-1-1	R	MR	MR	R	S	MR	MR	S	R	MR
14	TB356B-TB-47-3	MR	S	S	R	MR	R	R	MR	R	R
15	B11598C-TB-2-1-B-7	R	R	MR	R	S	R	MR	MR	R	R
16	B10553E-KN-68-1-1	MR	MR	S	R	S	MR	MR	S	R	R
17	BP1351D-1-2-PK-3-1	R	MR	MR	R	S	S	MR	S	S	R
18	B11604E-TB-2-3	MR	MR	–	R	MR	MR	MR	MR	MR	R
19	B11604E-TB-2-5	MR	MR	R	S	R	MR	R	MR	R	S
20	B11578F-MR-5-1	MR	MR	–	R	MR	R	–	S	R	MR
21	TB368B-TB-25-MR-2	R	S	MR	R	S	MR	MR	MR	MR	S
22	B11580E-MR-7-1-1	–	–	–	–	–	–	–	MR	MR	R
23	B11338F-TB-26	S	S	R	MR	MR	R	–	MR	S	R
24	B11587F-4-2	R	S	MR	MR	MR	MR	–	MR	R	R
25	TB490C-TB-1-2-1*	–	–	–	–	–	–	–	–	–	–
26	TB361B-30-6-2*	–	–	–	–	–	–	–	–	–	–
27	BP1976B-2-3-7-TB-1-1*	–	–	–	–	–	–	–	–	–	–
28	TB356B-TB-18-3*	–	–	–	–	–	–	–	–	–	–
29	IR30176*	–	–	–	–	–	–	–	–	–	–
30	IR60080-23*	–	–	–	–	–	–	–	–	–	–

* Has been cultivated by some farmers and performed blest resistance in the fields.
R = Resistant; MR = Moderately Resistant; S = Susceptible

Table 4 (continued)

No	Lines	Blast pathogen races								
		073	133	173	021	63	201	333	341	371
1	TB490C-TB-1-2-1-MR-4-29	R	S	S	R	S	R	R	R	S
2	TB490C-TB-1-2-1-MR-1-1	S	S	S	S	MR	S	R	R	MR
3	B11577E-MR-B-12-1	R	R	R	S	S	R	MR	R	MR
4	B11577E-MR-B-12-1-1	R	MR	R	R	MR	R	MR	R	MR
5	B11577E-MR-B-13-1-1-5-5	R	R	R	MR	R	R	S	S	MR
6	B11577E-MR-B-13-4	R	R	S	MR	S	S	R	MR	MR
7	IR65907-116-1-B-MR-4	R	R	R	MR	MR	S	R	S	S
8	B11602E-TB-1-2	MR	R	S	S	S	S	R	S	MR
9	B11602E-TB-1-3	R	R	S	MR	S	S	R	MR	MR
10	B11602E-TB-2-4-3	R	R	R	MR	R	R	MR	MR	S
11	TB409B-TB-14-3	R	MR	R	MR	S	R	MR	MR	MR
12	B11593-MR-11-B-2-8	S	R	R	MR	S	R	S	MR	MR
13	B10580E-KN-28-1-1	S	S	R	MR	R	R	R	R	MR
14	TB356B-TB-47-3	R	R	S	MR	S	S	R	MR	S
15	B11598C-TB-2-1-B-7	R	S	S	MR	S	MR	MR	MR	S
16	B10553E-KN-68-1-1	R	R	MR	MR	S	S	R	R	S
17	BP1351D-1-2-PK-3-1	R	R	S	S	S	S	R	MR	MR
18	B11604E-TB-2-3	MR	R	R	MR	R	R	R	R	R
19	B11604E-TB-2-5	S	R	R	MR	R	MR	R	R	R
20	B11578F-MR-5-1	R	R	MR	MR	R	R	R	MR	MR
21	TB368B-TB-25-MR-2	S	S	S	MR	MR	S	MR	MR	S
22	B11580E-MR-7-1-1	R	R	–	MR	R	MR	R	MR	R
23	B11338F-TB-26	R	R	R	S	R	MR	R	R	MR
24	B11587F-4-2	R	R	S	MR	R	MR	R	MR	MR
25	TB490C-TB-1-2-1*	–	–	–	–	–	–	–	–	–
26	TB361B-30-6-2*	–	–	–	–	–	–	–	–	–
27	BP1976B-2-3-7-TB-1-1*	–	–	–	–	–	–	–	–	–
28	TB356B-TB-18-3*	–	–	–	–	–	–	–	–	–
29	IR30176*	–	–	–	–	–	–	–	–	–
30	IR60080-23*	–	–	–	–	–	–	–	–	–

*Has been cultivated by some farmers and performed blast resistance in the fields.
R = Resistant; MR = Moderately Resistant; S = Susceptible

and 103–124 days maturity (Table 5). The lines were also rated as tolerant or moderately tolerant to Al toxicity. The tolerance to Al toxicity is an important character for the package since blast is more prominent in the wet regions where acid soils with the related nutrient problems including Al toxicity are distributed.

Consumer preferences to rice quality especially to cooked rice are differ across regions in Indonesia. Generaly, the people of Kalimantan island and West Sumatera prefer to cooked rice with hard texture, whereas the people of Java and most other regions prefer those with soft texture. Different packages, at least two with cooked rice textured hard and soft should be developed to meet the preferences of the consumers which is an important factor for adoption of introduced varieties. In this case, we are developing the package of the varieties with soft cooked rice texture. However, blast resistance diversity should be the major consideration for

Table 5 Some important characteristics of selected lines for developing package of 20 blast resistant varieties

No	Lines	Plant height (cm)	No. tillers/ hill	Maturity (days)	Al. Toxicity*	
					Veg.	Gen.
1	TB490C-TB-1-2-1-MR-4-29	148	9	115	T	T
2	TB490C-TB-1-2-1-MR-1-1	131	7	110	MT	MT
3	B11577E-MR-B-12-1	105	11	110	T	T
4	B11577E-MR-B-12-1-1	106	14	116	T	T
5	B11577E-MR-B-13-1-1-5-5	79	13	124	T	T
6	B11577E-MR-B-13-4	95	10	109	MT	MT
7	IR65907-116-1-B-MR-4	105	11	111	T	MT
8	B11602E-TB-1-2	79	12	117	MT	MT
9	B11602E-TB-1-3	74	12	113	T	MT
10	B11602E-TB-2-4-3	76	9	124	MT	MT
11	TB409B-TB-14-3	105	10	117	T	T
12	B11593-MR-11-B-2-8	112	9	116	MT	MT
13	B10580E-KN-28-1-1	93	8	119	MT	MT
14	TB356B-TB-47-3	87	8	104	T	MT
15	B11598C-TB-2-1-B-7	110	11	104	T	T
16	B10553E-KN-68-1-1	85	9	123	MT	MT
17	BP1351D-1-2-PK-3-1	106	9	117	MT	MT
18	B11604E-TB-2-3	100	12	113	T	T
19	B11602E-TB-2-5	71	7	122	MT	MT
20	B11578F-MR-5-1	88	12	117	MT	MT
21	TB368B-TB-25-MR-2	106	13	115	T	T
22	B11580E-MR-7-1-1	120	9	117	T	T
23	B11338F-TB-26	101	12	115	T	T
24	B11587F-4-2	93	8	103	T	T
25	TB490C-TB-1-2-1	140	10	119	–	–
26	TB361B-30-6-2	102	14	105	–	–
27	BP1976B-2-3-7-TB-1-1	109	13	109	–	–
28	TB356B-TB-18-3	120	10	110	–	–
29	IR30176	115	9	110	–	–
30	IR60080-23	114	10	110	–	–

* Scored during vegetative (Veg.) and generative stages (Gen.)
T = Tolerant; MT = Moderately Tolerant

selecting the varieties. Some lines with high amylose content related to hard rice texture, therefore, should be selected since there were no lines with soft rice texture resistant to blast race 373. Those lines were B11577E-MR-B-12-1, B11577E-MR-B-12-1-1, and B11577E-MR-B-13-1-1-5-5 (Table 6). All of the selected lines have good milled rice performances, medium – long grain length, medium – slender grain shape, and small – medium chalkiness.

A total of 30 selected lines are being evaluated in the farmers' fields for performance, farmer acceptability, and stability of the blast resistance. The blast resistance in the field should be depended on the resistance genes in the plant and the races composing the pathogen population, the two factors are also influenced by each other. Considering those interactions, the evaluation for the blast resistance stability

Table 6 Grain characteristics of upland rice selected for developing package of 20 blast resistant varieties

No	Lines	Amylose content (%)	Grain length	Grain shape	Chalkiness
1	TB490C-TB-1-2-1-MR-4-29	23,4	L	M	M
2	TB490C-TB-1-2-1-MR-1-1	22,3	L	M	M
3	B11577E-MR-B-12-1	25,3	M	M	M
4	B11577E-MR-B-12-1-1	28,7	L	M	S
5	B11577E-MR-B-13-1-1-5-5	26,4	M	M	M
6	B11577E-MR-B-13-4	23,7	L	M	M
7	IR65907-116-1-B-MR-4	18,3	L	M	M
8	B11602E-TB-1-2	23,0	L	M	M
9	B11602E-TB-1-3	19,8	M	M	S
10	B11602E-TB-2-4-3	25,3	M	M	M
11	TB409B-TB-14-3	23,2	L	S	S
12	B11593-MR-11-B-2-8	19,7	M	M	M
13	B10580E-KN-28-1-1	18,6	L	M	S
14	TB356B-TB-47-3	22,2	L	M	M
15	B11598C-TB-2-1-B-7	23,2	M	M	M
16	B10553E-KN-68-1-1	22,2	L	M	M
17	BP1351D-1-2-PK-3-1	22,6	L	M	M
18	B11604E-TB-2-3	24,1	L	M	S
19	B11604E-TB-2-5	23,6	L	M	M
20	B11576F-MR-5-1	18,1	L	M	S
21	TB368B-TB-25-MR-2	20,1	L	M	S
22	B11580E-MR-7-1-1	15,7	L	M	S
23	B11338F-TB-26	24,2	L	M	M
24	B11587F-4-2	21,3	M	M	M
25	TB490C-TB-1-2-1*	–	–	–	–
26	TB361B-30-6-2*	–	–	–	–
27	BP1976B-2-3-7-TB-1-1	21,2	L	M	S
28	TB356B-TB-18-3*	–	–	–	–
29	IR30176	22,5	M	M	S
30	IR60080-23	16,1	L	M	S

* Data not yet available

Grain length: L = long, M = Medium; Grain shape: M = Medium, S = Slender; Chalkiness: M = Medium, S = Small

of the selected lines is being conducted in wide areas of four to seven ha in four sites totalled about 20 ha. Based on the performances in the fields, 20 lines will be selected for the package which should be evaluated in wider areas and more sites before it is recommended for mass cultivation.

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Rice Blast in India and Strategies to Develop Durably Resistant Cultivars

Mukund Variar, C.M. Vera Cruz, M.G. Carrillo, J.C. Bhatt and R.B.S. Sangar

Abstract Analyses of the population of *Magnaporthe oryzae* at Hazaribag, India, revealed high pathogen diversity influenced by host cultivars, rice ecology and geographic location even though recent evidence suggested that the population is largely clonal. Host selection of low virulence frequency and unrestricted seed movement across the country help to introduce and establish new virulence in different regions. Strategies for blast management therefore focus on accumulation of race non-specific resistance genes in commercial cultivars susceptible to the disease. We evaluated a population of rice lines containing none to six defense response (DR) genes (thaumatin, oxalate oxidase, oxalate oxidase-like proteins, chitinase, peroxidase, HSP90) in three blast endemic locations during 2004–2006 and compared their performance with the level of resistance in monogenic lines having different *Pi* genes. The population was obtained by intermating advanced backcross derived lines of Vandana/Moroberekan (V_4M-14-1-B with V_4M-5-3-B, V_4M-6-1-B and V_4M-82-2-B). Disease progress curves in lines carrying five and six DR genes were comparable to the monogenic lines carrying R genes *Piz* and *Pi*9 effective at all three locations. While the monogenic lines generally exhibited an 'all or nothing effect' with high or low disease, the introgressed population had a range of disease intensities that declined progressively with the addition of each DR gene. Some defense response genes individually conferred a higher level of resistance compared to others and hence resistance was not proportional to the number of DRs present in all cases. Nevertheless, significant reduction in leaf blast intensity with increasing DRs in the introgressed lines at different locations and years suggested that accumulation of DRs conferring different mechanisms of resistance may contribute to non-specific resistance effective in multiple environments.

Keywords Defense response genes · *Magnaporthe oryzae*

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1 Introduction

Rice is grown in a wide range of agro-ecological conditions in India. Based on the harvested area of 42.8 million ha (Mha), about 55% of the rice crop is planted in Eastern India, 21% in the north, 18% in the south and about 6% in the west. There are several disease constraints to sustainable rice production, but generally the major ones common to all production areas are rice blast and bacterial blight. The relative losses from these diseases vary in the different production zones depending on the physical environment, crop management and pathogen population dynamics. The rice varieties grown in the different states are mostly location and ecosystem specific, primarily developed by the National Agricultural Research System (NARS) located in different states. Cultivars developed at IRRI and other International Agricultural Research System (IARS) have also found acceptance among farmers in India, some of them like IR36 and IR64 are widely grown all over the country.

With the introduction of modern varieties beginning with TN1, IR8 and Jaya, the area under irrigation increased and less frequent moisture stress reduced early and severe blast attack (Singh 1990). The consequent reduction in inoculum load and discontinued use of susceptible varieties led to a marked reduction in the area affected by blast whereas losses from bacterial blight have become more widespread. However, upland rice, grown in about 6.3 M ha in Eastern India and hill rice are more prone to blast and left uncontrolled because resistant varieties are not available and other management options are not remunerative. Blast causes more tonnage loss in lowland rice (14 M ha), especially in the cooler areas of Karnataka and the cool season crop in Andhra Pradesh, Tamil Nadu and Kerala, and management options that include host plant resistance and prophylaxis are commonly employed. In the basmati belt of north India, the economic loss due to blast is high because the quality of the harvested grain, other than actual yield loss, is also affected.

2 Blast Zones

Production-oriented survey (POS) is organized by the Directorate of Rice Research (DRR), Hyderabad every year to document constraints to rice production in various states of the country (Production Oriented Survey 1994–2006). Multi-disciplinary teams of scientists survey the crop during the season and report, among other constraints, the occurrence and intensity of major diseases in about 125 rice-growing districts in the country. Depending on the visual estimates of intensity of occurrence, diseases in a district are categorized as severe (S, >50%), moderate (M, 25–50%), low (L, 11–25%), or trace (T, <10%). Data available from POS reports for the period 1994–2006 was analyzed for the occurrence and intensity of blast by converting the categories (T, L, L-M, M, M-S, S) to scores (0, 1, 2, 3, 4, 5) for each surveyed district and averaged for different states (Variar 2006). Averaged over the 12 year period, Eastern India had the highest mean score (1.75), followed by north (1.53),

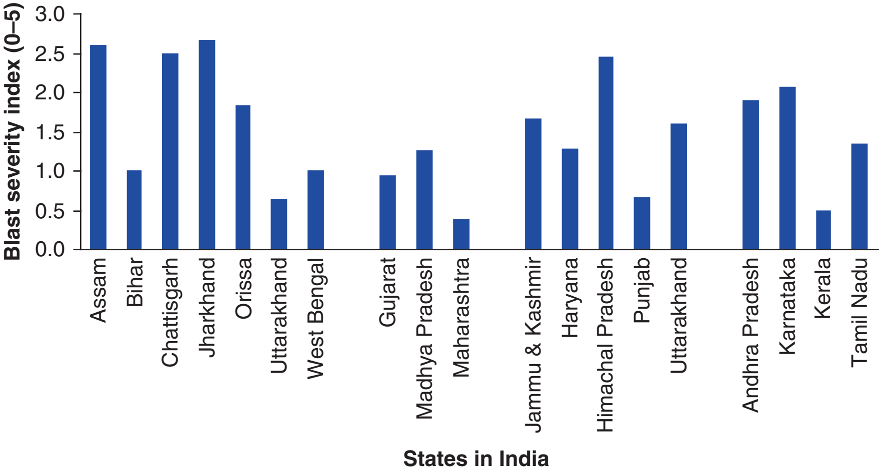

Fig. 1 Severity of blast in different states of India during 1994–2006. (Data source: Production Oriented Survey, DRR, 1994–2006)

south (1.44) and west (0.86). About 15% of the districts surveyed during 2001–2006 had moderate to severe blast. Severe blast was recorded in 1-2 districts each year. Wet season rice in the hills (Himachal Pradesh, Uttarakhand, Jammu & Kashmir) and plateaus (Jharkhand, Chattisgarh) of North and Eastern India scored higher than the plains in the same region (Fig. 1).

The rice varieties grown in the hills of Kashmir, Himachal Pradesh and Uttarakhand are cultivated with low N, but blast is widely prevalent under low to moderate intensity. In the Jammu region, high yielding varieties are grown at moderate fertilizer levels. Although blast occurs sporadically on these varieties, control measures are not taken. Current commercial cultivars grown in eastern India in rainfed uplands (Kalinga III, Vandana, Poornima) and lowlands (Mahamaya, Lalat, Swarna and Mahsuri) suffer some disease every year but losses are not severe in farmers' fields due to sub-optimal crop and nutrient management. Farmers cultivating quality rice in the North where Basmati types are highly susceptible limit N application to about 50 kg/ha to avoid severe losses. Blast was not a serious concern in the Indo-Gangetic plains (Punjab, Haryana, Uttar Pradesh, West Bengal) even though rice is grown under moderate to high N fertilizer levels during the south west monsoon season. The 'boro' season crop is reported to be affected by moderate blast in eastern Indian states.

Introduction of susceptible varieties like IR50 in the eighties did cause concern in South India but gradual replacement of this variety has arrested further episodic events even though varieties like BPT 5204 (Andhra Pradesh), ADT 43, ASD 16, White Ponni (Tamil Nadu), MTU 1010 and Intan (Karnataka) suffer moderate blast in different districts. N fertilizer application above the recommended doses (150 kg/ha in parts of Andhra Pradesh), cooler climate in the table lands of Karnataka during the South West Monsoon season and the second rice season in Tamil

Nadu (North East Monsoon), and preference for susceptible fine-grained varieties predispose the rice crop to blast in these states. The western Indian states (Maharashtra, Gujarat) have not reported blast to be a problem in recent years. Varieties developed in blast-free locations like Nawagam in Gujarat are, however, severely affected by blast when they are tested in blast endemic locations like Hazaribag indicating that rather than varietal response, it is the prevalent weather factors that appear to limit disease in Western India. Replacement of susceptible traditional varieties by modern varieties harboring one or several resistance genes, consequent reduction in inoculum load, and prevalence of relatively higher temperatures during the main crop season apparently limit blast incidence in the Indo-Gangetic plains and the deltaic zones of other major rivers in South India.

The survey reports indicate that in general, the high yielding varieties except IR 50 and a few others are less affected by blast than the traditional varieties even though these varieties are not specifically bred for blast resistance. Many of them are susceptible to blast but escape severe infection when grown in less blast-conducive environments. IR 36 and IR 64 contain the *Pita* gene, and IR 64 also has another closely linked gene *Pi20*. In addition to these known *Pi* genes, these varieties have accumulated several defense genes with their complex lineages (Ramalingam et al. 2003) which make them durably resistant in most locations. These varieties are used extensively in breeding programs in the southern states where blast is recognized as a potential threat to increased productivity. It is also recognized that with increase in crop intensification blast will be a potentially explosive problem in most of the eastern Indian states and North West hills. These states have reported state average yields reaching around 2–2.5 t/ha and the current yield gaps range from 37–70% in different states (Siddiq 2000).

3 Pathogen Variability

The data from POS clearly indicate that blast is a major constraint in certain agro-ecological regions where favorable weather conditions prevail. These reports, however, do not clarify the variability of the pathogen in the different regions. Reaction of three sets of differentials (Indian, Japanese, international), monitored in blast nurseries during 1980–1988 at Hazaribag was consistent (Variar 1986; Variar and Maiti 1988) with Raminad Str 3 and Zenith showing resistant reaction, and others among the international differentials showing susceptible reaction. Among the Japanese differentials, Fukunishiki and *Pi4* were resistant. The Directorate of Rice Research later initiated a program on monitoring the virulence of *M. oryzae* in a coordinated set up aimed to detect changes in the virulence pattern in the country over a period of time. Interestingly, there was no indication of any change in pathogen virulence in most of the locations, as per the reports for 2003–2006. During the wet season of 2005, a set of monogenic differentials made available through INGER was evaluated in replicated nurseries at three locations: Almora in the mid-Himalayas, Ambikapur and Hazaribag in the eastern Indian plateau region.

Table 1 Reaction of monogenic differentials to leaf blast at three locations in India, WS 2005

No.	Differentials	Gene	Ambikapur			Almora			Hazaribag		
			SES*	LN*	DLA*	SES	LN	AUDPC*	SES	LN	PBS*
1	IRBL1-CL	*Pi1*	9.0	66	50.0	7.0	6.6	19.8	8.0	191	55
2	IRBL11-ZH	*Pi 11*	9.0	66	75.0	5.5	2.7	5.1	5.7	61	3
3	IRBL12-M	*Pi 12*(t)	9.0	59	62.5	7.5	6.6	22.4	9.0	236	29
4	IRBL19-A	*Pi 19*	9.0	59	75.0	7.0	7.1	23.3	7.0	54	56
5	IRBL20-IR24	*Pi 20*	8.0	58	62.5	6.5	5.4	13.2	7.0	50	9
6	IRBL3-CP4	*Pi 3*	9.0	62	75.0	6.5	7.1	22.4	8.8	174	41
7	IRBL5-M	*Pi 5*(t)	9.0	69	75.0	4.5	1.2	1.2	3.0	55	25
8	IRBL7-M	*Pi 7*(t)	9.0	63	62.5	7.0	4.4	22.7	8.8	186	100
9	IRBL9-W	*Pi 9*	8.0	62	37.5	5.5	2.5	6.7	0.5	0	0
10	IRBLA-A	*Pi a*	9.0	75	62.5	7.0	6.0	15.7	8.5	184	59
11	IRBLA-C		4.0	16	3.0	8.0	9.8	33.8	8.0	250	0
12	IRBLB-B	*Pi b*	9.0	69	75.0	7.0	8.6	26.4	8.8	180	29
13	IRBLI-F5	*Pii*	9.0	63	38.0	7.0	8.9	25.8	8.3	122	79
14	IRBLK-KA	*Pi k*	9.0	67	50.0	6.5	6.1	13.4	7.3	106	58
15	IRBLKH-K3	*Pi k-h*	9.0	63	62.5	7.5	8.0	30.4	8.0	125	44
16	IRBLKM-TS	*Pi k-m*	9.0	66	62.5	7.0	5.0	15.2	5.5	31	62
17	IRBLKP-K60	*Pi k-p*	9.0	59	75.0	7.0	7.5	18.2	7.5	135	10
18	IRBLKS-F5	*Pi k-s*	9.0	65	75.0	7.0	7.5	15.6	9.0	199	59
19	IRBLKS-S		9.0	71	50.0	4.0	1.0	1.1	4.8	27	23
20	IRBLSH-B		9.0	27	25.0	5.0	1.2	2.2	2.8	13	38
21	IRBLSH-S	*Pi sh*	9.0	68	62.5	5.0	2.4	6.1	4.3	37	30
22	IRBLT-K59		9.0	76	50.0	6.5	6.7	10.8	7.3	54	88
23	IRBLTA-CP1	*Pi ta* (*Pi 4*)	9.0	70	75.0	5.0	2.3	3.6	9.0	300	79
24	IRBLTA-CT2		9.0	68	62.5	4.5	1.1	1.1	8.8	189	48
25	IRBLTA-K1		9.0	63	62.5	2.5	0.4	1.9	7.5	105	30
26	IRBLTA2-PI	*Pi ta-2*	9.0	64	62.5	2.0	0.7	0.6	1.0	4	0
27	IRBLTA2-RE		9.0	55	75.0	5.5	3.5	5.9	1.0	1	17
28	IRBLZ-FU	*Pi z*	4.0	21	3.0	4.0	0.9	1.2	1.5	2	0
29	IRBLZ5-CA	*Pi -5* (*Pi 2*)	3.0	6	1.0	5.0	2.1	4.9	1.5	3	6
30	IRBLZT-T	*Pi z-t*	9.0	63	62.5	7.0	9.8	27.1	8.5	217	94

*SES scale: 0–9; LN: Lesion Number at 30 DAS; AUDPC: Area Under Disease Progress Curve; PBS: Panicle Blast Severity (%).

Monogenic lines (MLs) bearing *Pia, Pib, Pii*, different alleles of *Pik, Piz-t, Pi3, Pi4, Pi7 and Pi12 (t)* were highly susceptible at all locations while *Pi2* and *Piz* were resistant (Table 1). Several MLs showed partial resistance at Almora and Hazaribag indicating that a part of the pathogen population was not virulent on *Pish, Pi5, Pi11, Pi19* and *Pi20*. The pathogen population at Ambikapur appeared to be composed of pathotypes virulent on most MLs including *Pi9* and *Pita-2* which were resistant at other locations.

Pathogen populations are generally characterized by virulence analysis on a set of differentials. Pathotyping or virulence analysis with a genetically well-defined set provides high degree of resolution for describing the virulence structure of a population. Development of near-isogenic lines (NILs) carrying single genes in the background of CO39 facilitated further work on pathogen characterization. Dey

Table 2 Reaction of selected isolates on blast near-isogenic lines in the background of CO39 at Hazaribag (Adapted from Dey 2003)

No	Genotype	R gene	1998				1999			
			Susceptibility		DLA*	UBN	Susceptibility		DLA*	UBN
			No (Rep)	%	20 DAI	(0–9)	No	%	20 DAI	(0–9)
1	C101 LAC	*Pi 1*	0	0	0.0	1	0	0	0.0	0
2	C101 A51	*Pi 2*	0	0	0.0	2	0	0	0.0	0
3	C104 PKT	*Pi 3*(t)	11(22)	100	7.5	4	11(22)	79	20.0	8
4	C101 PKT	*Pi 4a*	11(22)	100	10.0	4	9(18)	64	20.0	8
5	C105 TTP-4-L23	*Pi 4b*	11(22)	100	17.5	4	12(23)	82	32.5	7
6	RIL 249	*Pi 5*(t)	3(5)	23	2.5	4	3(5)	18	9.0	5
7	RIL 29	*Pi 7*(t)	0	0	1.5	4	0	0	5.0	5
8	CO 39 (S check)	*Pi a*	11(21)	96	12.5	9	12(24)	86	20.0	8
9	RR 222-1 (R check)	?	0	0	0	0	0	0	0	0

*Mean %DLA 20 days after inoculation with 11 (1998) and 14 Indian isolates (1999).

(2003) evaluated 25 eastern Indian isolates on NILs carrying *Pi1, Pi2, Pi3, Pi4 a, Pi4b, Pi5(t), Pi7(t)*, and *Pi9(t)* at Hazaribag. All the isolates were avirulent on *Pi1(t), Pi2(t), Pi7(t)* and the resistant check RR 222-1 (genetic constitution not known) (Table 2). Most isolates were virulent on *Pi3*, $Pi4^a$, $Pi4^b$ and the susceptible check CO39. Inoculation failed in one of the replications on four differentials. Differential reaction was unambiguous on *Pi5(t)* with nearly 23% isolates being virulent during 1998 and 18% during 1999. The isolates therefore, could be grouped into two, based on their virulence on *Pi5(t)*. However, RIL 249 carrying *Pi5(t)* was later on found to contain several QTLs in addition to *Pi5(t)*. Two isolates failed to initiate infection on this genotype in one of the replications. When polycyclic disease development was allowed and disease scored 20 days after inoculation, RIL 249 developed significantly less disease compared to the susceptible check CO39. It is likely that the QTLs present in the NIL interfered with disease development in this case. Though all isolates were avirulent on *Pi7(t)*, the NIL developed some disease 20 days after inoculation. This might be due to contamination as the inoculated trays were moved outdoor to allow polycyclic disease development. Susceptible reaction of *Pi7(t)* in the UBN subsequently confirmed that pathotypes virulent on *Pi7(t)* is present in the local population. Information gained from the inoculation tests on NILs indicated its usefulness in designating the isolates into discrete pathotypes but differential reaction was negligibly low with this set of NILs.

Detection and use of a variety of neutral markers distributed randomly in the *M. oryzae* genome has also permitted characterization of the fungal population in recent years. The highly repetitive element MGR 586 (contained in the plasmid pCB 586) has been particularly useful in analyzing genetic relatedness among blast isolates (Hamer et al. 1989). Analysis of the fungal populations at a number of sites across the world suggested that blast isolates are composed of discrete groups representing genetically related lineages (Levy et al. 1993; Xia et al. 1994; Zeigler 1994).

Characterization of the pathogen population from eastern (Sridhar et al. 1999), southern (Sivaraj et al. 1998) and northern India (Kumar et al. 1999) indicated that the population consists of site specific as well as widely distributed lineages. Kumar et al. (1999) reported that out of 45 lineages detected in 1992–1993 collection of 222 isolates from Indian Himalayas and Indo-Gangetic plains, 31 (69%) were site specific representing 18% of the isolates sampled. Among the 14 lineages (82% of the isolates) found at more than one site, four were detected at widely separated and geographically distinct sites. Lineages IHR 10 and IHR 11 were detected at 48 and 31%, respectively of the sites sampled. The two lineages represented 30% of the isolates in the collection, suggesting a strong clonal contribution to population structure. Host limitation and genetic isolation is reported from the Himalayas but there is also cumulative evidence that recombination affects population structure and dynamics of *M. oryzae* in this region (Kumar et al. 1999). Study at Hazaribag and Cuttack using blast isolates from several eastern Indian states revealed that the fungus is indeed highly diverse (Dey 2003). Based on fingerprint profiles, 19 lineages (RFLP, MGR 586) were recognized from 51 isolates and 38 lineages (PCR *pot2*) from 62 isolates at 75% similarity coefficient. Chadha and Gopalakrishna (2005) studied genetic variability and relationships among 20 Indian isolates of the fungus using RAPD markers. Analysis revealed high level of polymorphism, indicating a wide and diverse genetic base. It would appear that although most lineages are geographically isolated, migration also has occurred in some cases, possibly by seed transmission. Mishra et al. (2005) analysed *M. oryzae* isolates obtained from traditional and modern varieties in farmers' fields during three epidemics (1997, 2000 and 2002) in Orissa using *Pot 2* primer sequences. They found that traditional rice varieties were infected by a single predominant lineage of the pathogen during epidemics, whereas modern rice cultivars were infected by multiple lineages.

4 Breeding for Resistance

Breeding programs in the hills, plateaus and other blast-conducive environments normally use known blast resistance donors as one of the parents and screen the fixed populations for resistance to blast in uniform blast nurseries. Specific breeding programs with blast resistance as its major objective, as in the case of rainfed uplands, expose the F_2 single plants and F_3 families to blast first in the nursery and the resistant plants/families are selected subsequently for other agronomic/productive traits. Advanced breeding lines originating in the state agricultural universities and rice research institutes of ICAR from location/ecology specific breeding programs are evaluated in national coordinated trials across the country in outdoor blast nurseries at hotspots. The advanced breeding lines for different situations enter the coordinated set up as Preliminary Variety Trials (PVTs), selections from which are promoted to Advanced Variety Trials (AVTs). National screening nurseries for different biotic stresses, including blast, are composed of entries in PVTs (NSN2) and AVTs (NSN1). There are separate screening nurseries for breeding lines originating

Table 3 Distribution of genotypes in blast susceptibility classes in national screening nurseries (NSN) at Hazaribag, WS 2005

Score	Number of genotypes		
	Germplasm	NSN2*	NSN1*
0	22	56	44
1	7	13	4
2	10	37	10
3	41	12	6
4	179	120	26
5	269	110	15
6	379	79	7
7	254	31	3
8	17	28	3
9	0	4	0

*National screening nurseries for different biotic stresses, including blast, are composed of entries in Preliminary Variety Trials (NSN2) and Advanced Variety Trials (NSN1).

from hills (NSN-H) and hybrid rice (NHSN). A cursory glance at the frequency distribution of blast resistance in germplasm, NSN2, and NSN1 in one of the hotspots (Table 3) reveal that while it is skewed in favor of susceptibility in the germplasm and normally distributed in NSN2, the distribution is skewed in favor of resistance in NSN1. Highly susceptible lines are obviously eliminated in the selection process though several moderately susceptible but highly productive lines get promoted and eventually released for specific situations/ecologies for which they are primarily developed.

Unlike more intensive rice culture systems of Southeast Asia where emergence of new races have rendered resistance ineffective in newly released varieties within 2–3 years, India has not witnessed severe losses due to blast in recent years. The rice crop is, however, predisposed to infection due to favorable temperature regimes and intermittent drought in rainfed environments in hills and plateaus where many susceptible traditional varieties are not yet replaced by modern varieties in the different sub-ecologies. Yields are also constrained from reaching their potential due to increased leaf and neck blast infection under better nutrient management. Moreover, lineage analyses indicated that the pathogen is highly diverse and many lineages/pathotypes are present in natural populations. It is possible that host selection for virulence present in low frequencies eventually will render the resistance ineffective over a longer period depending on selection pressure. Analysis of the population obtained from traditional and high yielding varieties affected by blast epiphytotics in Orissa (Mishra et al. 2005) clearly indicated that high yielding genotypes harbored many lineages compared to the traditional varieties which were infected by a single predominant lineage. Conventional breeding and phenotyping is inefficient when several lineages of the pathogen are present and resistance to multiple lineages are warranted.

Indirect selection methods using molecular markers have helped in tracking and combining desirable genes in to common genetic backgrounds. Several laboratories

in India have identified closely linked markers for major genes using a variety of markers and fine mapping methods in several mapping populations (Naqvi et al. 1995; Hittalmani et al. 2000; Sharma et al. 2005). Identification and use of major genes for resistance has, however, not led to broad-spectrum durable resistance. Resistance has been especially short-lived in temperate ecosystems. Major obstacles to develop broad-spectrum durable blast resistant varieties is the spatial and temporal variability of the pathogen and host selection for virulence that are prevalent in low frequencies. Durability of resistance is also dependent on the type of resistance added to the host genotype and rice ecology which influences epidemiological potential of blast beyond general climatic differences, but more so, microclimatic differences. It was also suggested that durability or non-durability of resistance of a cultivar can also be due to cryptic error (insufficient range of the pathogen population sampled by the line where the resistance was evaluated) in the selection process in varietal development (Buddenhagen 1981).

The 'breakdown' of major gene resistance activated research to develop strategies to reduce future pathogen evolution. Deployment of '*R*' genes by rotation has been suggested but such precision farming techniques have limited scope in traditional farming systems as the genotypes possessing desirable *R* gene combinations need to be developed and deployed based on the predictive analysis of pathogen population dynamics. Detection and use of partial resistance to blast was emphasized with the hope that such resistance would be race non-specific, and therefore broad-spectrum and durable. Buddenhagen (1981) suggested that for durability, (1) vertical genes may be pyramided in a logical and measurable manner, if many are not allelic and (2) minor and major genes may be accumulated to reduce r (apparent infection rate) preferably in a recurrent selection system, and in an environment in which r is real and detectable. Development of horizontal resistance to blast was vigorously pursued at Institut Researche Agronomique Tropicale (IRAT) in the Ivory Coast (Notteghem 1993). Landraces (upland cultivars) with durable resistance under most blast-conducive conditions were identified. The new varieties developed also appear to have similar properties. Many of them like Moroberekan, OS 6, IRAT 13 have broad spectrum resistance in wide geographic regions including South and Southeast Asia. Though selection for partial resistance appeared to be possible using this approach, pyramiding of major genes or accumulation of minor genes appeared difficult mainly due to the masking effect of other genes.

5 Linking Gene Detection with Varietal Development

Wang et al. (1994) applied molecular mapping to investigate the genetic basis of durable resistance in Moroberekan, a traditional cultivar from Ivory Coast. Analysis of the recombinant inbred population derived from a cross between CO39 and Moroberekan showed that durable resistance involves a complex of genes associated with both partial and complete resistance. Since assessment of the effects of various putative resistance loci over sites would be useful for gene deployment, QTL/E

effects were analysed using this population in the uplands of Eastern India. Resistance to blast was evaluated in 180 RILs in the wet seasons of 1997 (Hazaribag), 1998 (Almora, Ambikapur and Hazaribag) and 1999 (Hazaribag). QTL mapping was achieved by singe marker analyses (127 markers) and regression on flanking markers using BSTAT statistical software. A locus in the interval between RG 449 and RG 788 on chromosome 4 had the maximum individual influence on complete and partial resistance traits. This could be *Pi5(t)*, which was identified with certainty in earlier studies in inoculation tests as well as under field conditions in the Philippines and Indonesia (Wang et al. 1994). Two other genes were identified to have independent effects on IT. They were mapped to a chromosome segment between RG 528 and RG 272 on chromosome 7 and between RG 181 and RG 323 on chromosome 12. Several other loci had lower individual effects on infection type but complemented each other to ensure complete resistance as revealed by interaction analysis. A number of markers (QTLs) affecting lesion number, lesion size and diseased leaf area were mapped on the same chromosomal regions (Chromosome 6, 11 and 12) which were previously identified to have complete resistance genes. Though the results support the hypothesis that qualitative genetic loci are the same loci that affect quantitative traits (Beavis et al. 1991), the presence of gene constellations influencing both complete and partial resistance appears more likely. Comparison of the results with those of Wang et al. (1994) and Yu et al. (1991) suggest that there may be more than one gene influencing complete and partial resistance near RG 16 on chromosome 11 and RG 181 on chromosome 12. *Pi7(t)* (near RG 16 towards RG 103A) was identified to have major influence on complete and partial resistance in the Philippines (Wang et al. 1994). There was no evidence for a role of *Pi7(t)* in reducing infection type or DLA at Hazaribag but the QTL affecting partial resistance near this locus (located between RG 16 and CDO 365) was effective in reducing DLA in the Philippines and India. Some of the QTLs detected in this population were effective at all test locations. Many of them were common to those detected earlier in the Philippines and Indonesia.

Markers associated with complete resistance were also mapped in the Azucena x Bala population based on phenotyping of 110 RILs at these locations. A combined RFLP and AFLP map with 134 markers were used to detect QTLs associated with infection type, lesion number, lesion size and diseased leaf area. There were only two segments (G 1010, chromosome 8; R 1933, chromosome 12) with major QTL effects at a threshold of 0.01 %. The QTL at or near the marker R 1933 was associated with lesion size only whereas G 1010 was associated with lesion type, number, size and DLA. One of them was derived from Azucena (R 1933) and the other from Bala (*Pi33*). The results across locations showed minimum QTL/environment interaction indicating that QTLs will be effective across locations.

Simultaneous detection of major genes and/or QTLs for blast resistance and their transfer to elite backgrounds would accelerate the process of varietal development. Since the blast resistance QTLs were all contributed by Moroberekan in the CO39 × Moroberekan population, it was used in hybridization to improve blast resistance in an upland rice variety Vandana, popular in the Eastern Indian uplands. Advanced backcross method was used (Wu et al. 2004). Seven QTLs were identified

($P < 0.0001$) when BC_2F_4 and BC_3F_4 populations were analyzed together; two QTLs contributed by Vandana (RM 297, chromosome 1; OSR 32, chromosome 12) and four contributed by Moroberekan (RM 259, chromosome 1; RM 261, Chromosome 4; OSR 22, chromosome 7; RM 144, chromosome 11) were associated with leaf blast, one QTL derived from Moroberekan (RM 204, chromosome 6) was associated with panicle blast severity (Variar et al. 2005). The QTLs derived from Vandana were not effective at Almora but effective at all other locations (Hazaribag, Jharkhand; Ambikapur, Chattisgarh; Santapur, Orissa). This was expected as the recurring parent Vandana was susceptible at Almora and moderately resistant to leaf blast at other locations.

Quantitative, race-nonspecific resistance against blast is desirable but it is difficult to accumulate quantitative resistance without the knowledge of the underlying genetic control and corresponding mechanisms involved in resistance expression. Recent work has shown that selection for candidate defense response genes (CGs) is effective in accumulating a high level of quantitative resistance. The level of resistance was proportional to the number of candidate gene alleles accumulated in advanced breeding lines and the resulting resistance was race non-specific and effective in multiple environments. Four BC_3F_4 lines of Vandana/Moroberekan carrying different candidate genes (thaumatin, oxalate oxidase, oxalate oxidase-like proteins, chitinase, peroxidise, HSP90) and phenotyped for blast resistance were intermated at IRRI (Table 4) and the progenies (which attained homozygosity within two years) with acceptable phenotype were field tested in several eastern Indian locations for blast resistance and productive/agronomic traits during 2004–2006. Disease progress curves in lines carrying five and six CGs were comparable to the monogenic lines carrying R genes *Piz* and *Pi9* effective at all three locations. While the monogenic lines generally exhibited an ‘all or nothing effect’ with high or low disease, the introgressed population had a range of disease intensities that generally declined progressively with the addition of each CG (Fig. 2). Some defense response genes individually conferred a higher level of resistance compared to others and hence resistance was not proportional to the number of CGs present in all cases. Nevertheless, significant reduction in leaf blast intensity with increasing CGs in the introgressed lines at different locations and years suggested that accumulation of CGs conferring different mechanisms of resistance may contribute to non-specific resistance effective in multiple environments. QTL analysis using advanced backcross populations suggested that relatively few chromosomal regions (5–10) can confer adequate resistance indicating that it is feasible to reconstitute the level of resistance as seen in the parents (Leach et al. 2007). Some candidate defense genes, notably members of the gene families of oxalate oxidases (OsOXO) in chromosome 3 and oxalate oxidase-like proteins (OsOXLP) in chromosome 8 are consistently associated with blast resistance (Carrillo et al. 2005; Davidson et al. 2006). Advanced backcross method can be gainfully used for simultaneous detection of QTLs and varietal development with one or two additional matings to accumulate the favorable alleles. The time required for attaining homozygosity is considerably less in case of intermating with advanced backcross derived lines.

Table 4 Candidate defense response genes associated with blast resistance in parental lines derived from Vandana/Moroberekan population and intermated to accumulate different mechanisms of blast resistance, IRRI 2002

Parental lines and donors	Chitinase	Ox-Ox	HSP90	Aldose red.	Thaumatin	Peroxidase	Ox-ox-like	PR1	DPA	PR10	#CGs
M'berekan	+	+	+	+	+	+	+	+	+	+	10
Vandana	–	–	–	–	–	–	–	–	–	–	0
VM14-1-B	–	+	+	–	+	+	–	+	–	–	5
VM 5-3-B	–	–	–	–	–	–	+/–	–	–	–	1
VM 6-1-B	–	–	–	–	–	–	+	–	–	–	1
VM 82-2-B	–	+	–	–	–	–	+/–	–	+/–	–	3

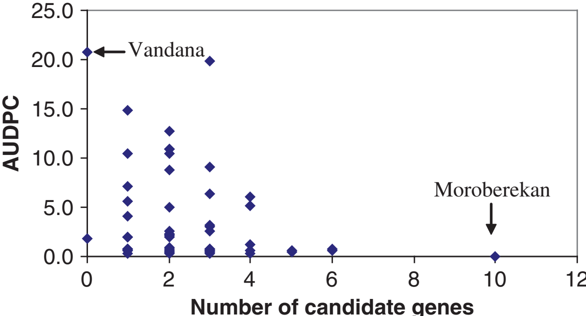

Fig. 2 The Area Under Disease Progress Curve (AUDPC) evaluated for blast resistance in intermated lines derived from Vandana/Moroberekan population declined progressively with addition of each candidate defense response gene

It is obvious that the main issue is durability, for which identification and deployment of both qualitative and quantitative resistance is needed, preferably the first superimposed over the latter. Genetic mapping as a prerequisite to genetic analysis is now part of standard plant breeding. Base molecular map is now available for rice with a sequenced map of $> 40,000$ SSRs and SNPs. QTL association mapping can now be done to identify those regions where segregation of the trait is associated with segregation for the markers. Transfer of the improved trait to an already otherwise adapted variety can proceed simply by selecting for and accumulating favorable alleles. However, the expression of the traits themselves still poses a problem. While genotyping has progressed well, phenotyping has been restricted mostly to glasshouse environments which do not often differentiate the lines sufficiently to enable reliable detection of associated QTLs. Further work is needed not only to combine different resistance genes but also place them in productive backgrounds as farmers are unlikely to abandon cultivation of preferred varieties like Mahsuri, Samba Mahsuri or traditional basmati types in favor of resistance alone. Yield penalties associated with resistance is still not definitely known even though few examples like IR 36 and IR 64 indicated that it is possible to combine resistance with productivity.

6 Integration with Other Management Options

Strategies for blast management would vary in different production zones. Combining blast resistance with such resistance enhancers like biogenic silica, SPAD/LCC-based N nutrition, and mitigation of drought effects will have immediate usefulness in rainfed environments. When blast incidence is limited to the nursery in the boro season, seed treatment with tricyclazole would be enough but if the disease tends to spread to the main plot causing economic damage frequently in a locality, disease management with host plant resistance should be ideal. It is also possible that the shorter duration modern varieties deployed in the boro season would also be grown in the aus season. Under such conditions, the genotypes need at least moderate resistance against blast. Efficacy of chemicals or natural plant products will also be higher when used in conjunction with moderate levels of resistance. A recently

introduced Nativo™ 75 WG which, when applied in a range of 120–150 g active ingredient/ha, controlled most of the major rice diseases. This new fungicide is a balanced mixture of two active ingredients with different modes of action: the triazole Tebuconazole, an ergosterol synthesis inhibitor and Trifloxystrobin, a strobilurin of the new generation inhibiting electron transport across the mitochondrial membrane. The different behaviour of tebuconazole and trifloxystrobin combining penetration and redistribution gives Nativo™ 75 WG the potential to control a broad spectrum of diseases at different stages of infection.

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Breeding Rice Cultivars with Durable Blast Resistance in Colombia

Fernando Correa Victoria and Cesar Martinez

Abstract Rice blast disease (*Pyricularia grisea*) is the most important rice production constraint in Latin America. One strategy to improve the durability of blast resistance is to pyramid resistance genes. To do this, we have conducted extensive studies on the genetic structure of blast pathogen populations in Colombia and Latin America; determined composition, distribution and frequency of the avirulences that underlie race variation; identified and incorporated resistance gene combinations into commercial rice cultivars using genetic markers; and continuously evaluated and selected breeding lines under high disease pressure and pathogen diversity. Rice differentials with known blast resistance genes have been used to study avirulence gene composition and frequency in the blast pathogen and to identify relevant resistance genes. The combination of the blast resistance genes (*Pi-1, Pi-2, Pi-33*) for which their corresponding avirulence genes are highly conserved in blast pathogen populations in Colombia has proven to confer stable blast resistance after several years of testing under high blast pressure in the field and greenhouse inoculations. Additional pathogen characterization of spontaneous mutations of the blast pathogen allowed the identification of the blast resistance genes *Pi-b, Pi-9 and Pi-ta*^{2}, which will be needed for protecting rice cultivars from potential future changes in the avirulence/virulence genes in the blast pathogen population. Microsatellite markers highly linked to these blast resistance genes have been found from public databases facilitating the introgression and pyramiding of each of these six blast resistance genes into Latin American rice cultivars and elite lines derived from rice breeding programs aiming at developing rice cultivars with durable blast resistance.

Keywords Durable resistance · Rice blast · Avirulence/virulence · Resistance genes

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1 Introduction

Rice blast caused by *Pyricularia grisea* (Cooke) Sacc., is the most limiting biotic factor of rice production in the world. The use of resistant cultivars is the most effective and economical way of controlling the blast disease, therefore, breeding efforts for developing resistant cultivars continue to be a priority of all rice breeding programs worldwide. Rice is normally grown under irrigation or flooded conditions characterized by low blast pressure or under rainfed upland conditions characterized by a high blast pressure (Bonman and Mackill 1988, Bonman et al. 1991). Development of durable blast resistance for these two environments should be possible if breeding programs are based on a complete understanding of pathogen diversity in the target area (Correa-Victoria et al. 2004). The Rice Project at the International Center for Tropical Agriculture, CIAT, has been developing a breeding strategy for the development of durable blast resistance. This strategy is based on studies on the composition and frequency of avirulence genes of the pathogen, characterization of the genetic structure, identification and incorporation of resistance gene combinations into commercial rice cultivars effective against populations of each genetic family of the pathogen, and the continuous evaluation and selection of breeding lines under a high disease pressure and pathogen diversity (Correa-Victoria et al. 2004).

2 Breeding Strategies for Durable Rice Blast Resistance

2.1 Irrigated Rice Production System Under Low Blast Pressure

CIAT's Rice Project develops its breeding activities on resistance to *P. grisea* under favored upland conditions in the experiment station "Santa Rosa" from FEDEARROZ in the Department of Meta, Colombia. This "hot spot" site is characterized by high blast disease pressure and pathogen diversity, which is maintained in the breeding plots during the entire crop cycle using spreader rows composed of a mixture of commercial rice cultivars susceptible to the different genetic lineages of the pathogen (Correa-Victoria et al. 2004). Under this condition of evaluation and selection, we have found that the resistance selected is more stable and durable than when resistance is selected under conditions of less blast pressure. Using this site and studies on the virulence diversity of the fungus, we have identified specific resistance gene combinations which explain the resistance stability of certain lines under irrigated conditions, indicating that pyramiding non-allelic major genes may be used to develop durable resistance to blast. The isogenic lines CT 13432-68, CT 13432-54, and CT 13432-55 carry the resistance genes *Pi-1, Pi-2, and Pi-33* respectively. These complementary resistance genes, in combination or when pyramided in the isogenic line CT 13432-107, confer resistance to all the main isolates representing the most common genetic families of the blast fungus in Colombia (Table 1). Our results have demonstrated that the combination of these three major blast resistance genes is higly effective for breeding and development of rice cultivars with durable resistance to the blast pathogen in Colombia. The basis of this

Table 1 Resistance genes relevant to genetic lineages of *Pyricularia grisea* in Colombia

Cultivar	Resistance Gene	Isolate/Genetic Lineage (L)								
		1 L6-1	2 L6-2	3 L6-3	4 L4-1	5 L4-2	6 L4-3	7 L5-1	8 L5-2	9 L5-3
CT 13432-68	Pi-1	S[1]	S	**R**	**R**	**R**	**R**	S	S	S
CT 13432-54	Pi-2	S	S	S	S	S	S	**R**	**R**	**R**
CT 13432-55	Pi-33	**R**[1]	**R**	**R**	S	S	S	S	S	S
CT 13432-107	**Pi-1 + 2 + 33**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**

[1] S=susceptible interaction; R=resistant interaction

resistance comes from our studies indicating that the frequency of the avirulence genes in the pathogen can be used as an indicator to predict the durability of a resistance gene. In other words, those resistance genes corresponding to avirulence genes whose loss during resistance breakdown causes a deleterious effect on the pathogen will probably be durable (Correa-Victoria et al. 2004).

The great pathogenic diversity observed in *P. grisea* is considered the main cause of resistance loss in newly released cultivars. All breeding programs aimed at developing rice cultivars with durable resistance should be based on the study and understanding of the genetic diversity of the pathogen, virulence diversity, and early detection of possible changes in avirulence/virulence during evolution of the fungus. We have conducted continuous monitoring of the blast pathogen population in Colombia to identify possible spontaneous mutants able to breakdown the three gene combination, in order to identify potential resistance genes effective against these mutations before these isolates increase in frequency and become predominant within the fungus population. Analysis of several hundred isolates demonstrated the effectiveness and stability of the three resistance gene combination; however, three isolates (Table 2) defeated the combination of the resistance genes in the isoline CT 13432-107 as well as each individual gene (isolates 1, 2 and 3, Table 2) in greenhouse inoculations. Although these isolates were detected in very low frequency and it is not known their potential fitness to become predominant in nature, we used them for inoculating rice differentials with known blast resistance genes to identify potential resistance genes effective against these three isolates. These inoculations allowed the early identification of the resistance genes *Pi-9, Pi-b, and Pi-ta*[2], which

Table 2 Identification of resistance genes to blast isolates compatible with the resistance genes Pi-1, Pi-2 and Pi-33

Rice Differential	Resistance Gene	Isolates							Avirulence Frequency
		1	2	3	4	5	6	7	
CT 13432-68	Pi-1	S	S	S	S	**R**	S	S	0.53
CT 13432-267	Pi-2	S	S	S	S	S	**R**	**R**	0.16
CT 13432-33	Pi-33	S	S	S	**R**	S	S	S	0.18
F 145	Pi-b	S	**R**	S	S	S	S	S	0.40
F 128-1	Pi-ta[2]	S	S	**R**	S	S	S	**R**	0.15
75-1-127	Pi-9	**R**	**R**	**R**	**R**	**R**	**R**	S	0.98

are effective against the blast isolate mutants 1, 2, 3, respectively. Therefore, the newly identified resistance genes should also be incorporated into future rice cultivars in order to remain ahead of possible changes in virulence within the blast pathogen population.

The resistance gene *Pi-9* derived from the wild species *Oryza minuta* was found to be effective against the isolates 1, 2, and 3 as well as the isolates 4, 5 and 6. These last three isolates represent the genetic lineages SRL-6, SRL-4, and SRL-5, the predominant genetic lineages from Colombia, respectively. The resistance genes *Pi-ta*^{2} *and Pi-b* are not effective against isolates 4, 5 and 6. Although the resistance gene *Pi-9* is effective against the isolate mutants 1, 2 and 3, and the isolates representing the most common lineages of the pathogen, this gene should not be used as a single gene because of the high risk of a rapid breakdown. The avirulence frequency of 0.98 for the resistance gene *Pi-9* (Table 2) is considerable high compared to the frequencies found for the other resistance genes (Table 2). However, a few isolates were found to be fully compatible with this gene. Fortunately, the resistance genes *Pi-ta*^{2} *and Pi-2* are effective against this pathotype (isolate 7, Table 2). *Pi-9* has been reported to exhibit a broad spectrum of resistance to many blast populations of the world (Qu et al. 2006); however, this gene should be used in combination with other resistance genes to avoid a rapid breakdown of the resistance. It is interesting to note that although the resistance genes *Pi-2 and Pi-9* are in the same gene cluster, isolates compatible with *Pi-2* do not infect *Pi-9*, and the isolate compatible with *Pi-9* do not infect *Pi-2*. In summary, development of durable blast resistance for the irrigated rice ecosystem in Colombia should be based on the combination of the resistance genes *Pi-1, Pi-2, Pi-33, Pi-b, Pita*^{2}, and *Pi-9*, which in combination are effective against the present blast populations.

With the objective of identifying sources of the six blast resistance genes of interest, more than 200 commercial rice cultivars from Latin America were inoculated with different blast isolates carrying the corresponding avirulence gene to each one of the six blast resistance genes. The presence of the genes and their frequency in the germplasm evaluated were inferred from the phenotypic evaluations (Table 3)

Table 3 Frequency of blast resistance genes in 211 commercial rice cultivars from Latin America

Resistance Gene	Cultivars No.	Frequency	Resistance Gene	Cultivars No.	Frequency
Pi-9	**12**	**0.06**	Pi-4b	69	0.33
Pi-k^a	35	0.17	Pi-3	83	0.39
Pi-t	46	0.22	Pi-ta	94	0.45
Pi-z^t	48	0.23	Pi-k	99	0.47
Pi-k^h	55	0.26	**Pi-b**	**112**	**0.53**
Pi-k^m	55	0.26	Pi-i	117	0.55
Pi-z	62	0.29	Pi-4a	125	0.59
Pi-k^p	64	0.30	**Pi-ta^2**	**137**	**0.65**
Pi-1	**64**	**0.30**	Pi-k^s	158	0.75
Pi-33	**64**	**0.30**	Pi-sh	178	0.84
Pi-2	**68**	**0.32**			

and will be confirmed by the use of molecular markers. Sources of each gene to be used in genetic crosses were found among the cultivars evaluated being already present in backgrounds with other desirable agronomic traits. The frequency of the six genes of interest was between 6 (*Pi-9*) and 65 (*Pi-ta*^{2}) percent. Pyramiding blast resistance genes in a common background can be assisted by the use of molecular markers linked to the resistance genes. Several microsatellite markers associated to these six blast resistance genes have been reported and are currently used at the rice breeding program at CIAT (Correa-Victoria et al. 2006, Fuentes et al. 2007) with the objective of developing rice cultivars with durable blast resistance for irrigated rice ecosystems.

2.2 Favored Upland Rice Production System Under High Blast Pressure

Rice blast is a major constraint to rice production, particularly under the rainfed conditions prevalent in Latin America. Since many rice growers in developing countries do not have access to fungicides, cultivar resistance has been the preferred means of controlling the disease. Developing durably resistant lines, therefore, is a high priority for most rice-breeding programs working for this upland environment. As indicated above, the CIAT Rice Project develops its breeding activities on resistance to *P. grisea* under favored upland conditions in the Santa Rosa experiment station. This site is characterized for having high blast disease pressure and pathogen diversity. Correa-Victoria and Zeigler (1995), concluded that high levels of resistance selected from rice populations with diverse combinations of resistance genes in the presence of diverse and abundant pathogen population may yield lines with stable blast resistance.

Oryzica Llanos 5 was released from the CIAT Rice Breeding Program in 1989 as a modern, high yielding, and blast resistant cultivar for the very blast-prone eastern plains of Colombia. It was widely grown in this region and remains highly resistant after more than 15 years. This cultivar has shown a remarkable durability in resistance over space as well as over time. It had a virtually unmatched degree of resistance when evaluated in several highly blast-conducive sites in Asia (Correa-Victoria and Zeigler 1995). At that time, it was presumed that the resistance in the rice cultivar Oryzica Llanos 5 was most probably multigenic, because all its ancestors were susceptible to isolates from our breeding experiment station.

It has been suggested by many rice blast researchers that in environments highly conducive to the development of severe blast epidemics, such as favored uplands, partial resistance may offer adequate protection from the blast pathogen. However, selection for partial or quantitative resistance to rice blast is difficult because of its genetic nature where several minor genes are controlling it. Consequently, breeding for partial resistance usually involves simply selecting plants showing intermediate disease levels over several generations, discarding both highly susceptible and highly resistant plants. In addition, Correa-Victoria and Zeigler (1995) concluded

that rice lines selected with complete resistance at this experiment station were more stable than rice lines selected as partially resistant. They also concluded that developing high levels of multigenic resistance for disease-prone environments is an attractive and achievable alternative to partial resistance.

The genetic basis of the high level of durable resistance to rice blast in the cultivar Oryzica Llanos 5 was characterized in two recombinant inbred line (RILs) populations from a cross between the susceptible cultivar Fanny and Oryzica Llanos 5 (Lopez-Gerena 2006, Lopez-Gerena et al. 2004). The number and chromosomal location of quantitative trait loci (QTL) conferring resistance against eight isolates of the blast fungus representing different genetic lineages were tested in these two populations and a linkage map was constructed using 350 molecular markers. Twenty-one QTL were detected and associated with the resistant traits, disease leaf area and lesion type, on nine chromosomes. Most but not all of the QTL occurred in the same genomic regions as either genes with major race-specific effects or other resistance QTL that had been described in previous experiments. Most of the QTL appeared to be race-specific in their effects but it is possible that some QTL with smaller effects were nonspecific. Three QTL affected resistance to one blast isolate, which causes limited disease on Oryzica Llanos 5 in the greenhouse and was probably virulent on most or all of the major genes from the cultivar. As a whole, the observed durable resistance in the rice cultivar Oryzica Llanos 5 seems to be the result from a combination of quantitative and qualitative resistance genes. The information from these studies is being used for the development of improved lines with Oryzica Llanos 5 derived QTL for resistance. The growing number of mapped minor and major resistance genes and development of marker-aided selection suggests that quantitative blast-resistance genes may be efficiently combined with major genes.

Despite all the efforts made to improve the efficiency for selecting and developing rice cultivars with durable blast resistance for the favored upland rice ecosystem, blast resistance is continuously being lost in breeding lines after the F_4 or later generations. A long-term study (Tables 4 and 5) was conducted by selecting resistant plants in the F_2 populations of year 2000 at our experiment station to associate the stability of blast resistance in advanced generations and the reaction of the F_2 populations. All crosses were developed from progenitors which exhibited a stable blast resistance at the experiment station. F_7 lines derived from F_2 resistant plants selected

Table 4 Long term study on the stability of rice blast resistance under upland conditions and high blast pressure in Colombia (F_2–F_3 generation)

Population	Crosses No.	Families No.	Resistant F_2 plants Selected 2000	Resistant F_3 lines 2001		Resistant F_3 plants Selected 2001
				No.	%	
F_2 Family Field Reaction						
Susceptible	69	100	194	39	20	661
Segregating	72	96	171	53	31	698
Resistant	54	78	155	80	52	644
Total	**156**	**274**	**520**			**2003**

Table 5 Long term study on the stability of rice blast resistance under upland conditions and high blast pressure in Colombia (F_4–F_7 generation)

Population	Resistant F_3 plants Selected 2001	Resistant F_4 lines 2002		Resistant F_5 lines 2003		Resistant F_6 lines 2004		Resistant F_7 lines 2005	
		No.	%	No.	%	No.	%	No.	%
F_2 Family Field Reaction									
Susceptible	661	89	13	5	1	0	0	0	0
Segregating	698	166	24	36	5	25	4	22	3
Resistant	644	126	20	30	5	25	4	22	3
Total	**2003**	**371**	**19**	**71**	**4**	**50**	**3**	**44**	**2**

in 2000 were evaluated for several seasons. A high decline in the number of resistant lines was observed (Table 4). No line derived from F_2 families with a predominant reaction of susceptible plants (more than 50% susceptible plants) remained with stable blast resistance at the F_7 generation (Table 5). Although F_2 families with predominant segregation (half of the F_2 plants are resistant and half susceptible) or resistant plants (more than 50% of the F_2 plants are resistant) yielded stable blast resistant lines, this percentage was very low (3%) for both cases (Table 5). This percentage is considered significantly low given that the breeders normally have to evaluate several thousand lines in the F_3 generation. A total of 44 rice lines (Table 5) exhibited a stable blast resistant reaction over the years and these lines are being used for distribution in the region, as progenitors in future crosses, and for evaluation studies of the stability of the resistance on a yearly basis in replicated trials.

Our hypothesis in this study was to demonstrate that those lines originating in crosses, where the F_2 families show a higher number of blast resistant plants, and which showed a higher number of resistant sister lines, would give origin to more stable resistant lines in the advanced generations. In addition, those advanced resistant lines originating from F_2 resistant plants selected within crosses where F_2 susceptible plants predominate, would be less stable. Our reasoning behind this hypothesis is that F_2 populations exhibiting a predominant number of resistant plants carry a larger number of different resistant genes including major and minor genes. Advanced resistant lines originating in these populations have a greater probability to carry a larger number of these resistance genes and therefore would be more stable. Those families with few F_2 resistant plants would probably have fewer resistance genes, and these would be easily defeated by the pathogen in early generations. If this hypothesis is correct, breeders should rate the F_2 populations and eliminate those crosses where the susceptible plants predominate allowing breeder's efforts to be concentrated on those crosses where there is a greater probability of selecting stable blast resistant rice lines. On the basis of our results in the F_6 and F_7 generations, our hypothesis seems to be true in relation to the development of stable blast resistant lines originating from F_2 populations where resistant plants predominate.

We should consider the important concern arising from the large decline in the number of resistant lines from one generation to the other. Our studies on the parents involved in the crosses evaluated suggest that some parents are better than others for potentially giving origin to stable blast resistance. We recommend that before incorporating new parents as sources of stable blast resistance in a breeding program, a careful evaluation over time and under high blast pressure be conducted. We also recommend selection of individual resistant plants in the F_3 generation. Plant selection in the F_2 generation is not an accurate indicator of resistance, because it is based on a single plant and not on a line composed of several plants that would better represent the range of resistance genes in the original F_2 plant.

Several potential sources of stable blast resistance (32 F7 resistant lines) were selected from our long-term study to be involved in the development of a modified recurrent selection program aimed at accumulating the different resistance genes controlling their stable resistance. A total of 32 progenitors were selected to initiate the development of the F_1 of 16 single crosses; these F_1 were used for the development of double crosses (2×2) repeating the process for the development of 4×4, 8×8, and 16×16 populations. Besides the stable blast resistance exhibited by each progenitor, other criteria such as yield potential, grain quality, adaptation to the upland ecosystem and tolerance to other diseases was considered. All crosses are being developed manually controlling the involvement of all 32 parents in the last cross (16×16). Resistant plants are being selected and advanced expecting to generate large numbers of stable resistant lines that could become commercial rice cultivars in the near future. We expect through recurrent selection to be able to accumulate both major and minor blast resistance genes conferring durable blast resistance. We expect from our other studies to use molecular markers associated with major genes to assure that selected lines carry them and expect in the near future to identify markers associated to relevant minor genes to assure that selected lines do not carry only major resistance genes.

3 Conclusions: Breeding Strategies for Durable Rice Blast Resistance

3.1 Irrigated Rice Production System Under Low Blast Pressure

- Pyramiding of major rice blast resistance genes based on targeted blast populations
- Breeding assisted by molecular markers and pathogen characterization (avirulence frequencies)

3.2 Upland Rice Production System Under High Blast Pressure

- Evaluation and selection of potential sources of stable blast resistance over time under blast hot spot conditions. Crosses among progenitors following a recurrent selection procedure

- Evaluation and selection of segregating populations under high blast pressure and pathogenic variation (hot spots)
- Selection of advanced rice lines exhibiting stable blast resistance over time combined with desired agronomic traits
- Marker assisted selection for major blast R genes

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What it Takes to Achieve Durable Resistance to Rice Blast?

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Abstract Based on performance record, varieties with a reputation for possessing durable blast resistance can be identified from breeding programs. For most of these varieties, a combination of major resistance genes and multiple QTL appears to be the underlying genetic basis for durability. Despite this seemingly simple observation, re-creating genotypes with durable resistance and proper agronomic characteristics remains challenging in breeding. We suggest that genetic mapping, saturation mutagenesis, and transcriptome analysis can together provide a genome-wide view of the essential regions for disease resistance, enabling the creation and selection of desirable gene combinations. We further propose actions needed to sustain breeding for durable resistance. These include (a) develop breeding-ready near-isogenic lines for important major resistance (R) genes and QTL, (b) improve knowledge of neck blast through better screening and genetic analysis, (c) promote exchange of breeding materials between countries for evaluation and validation of durable resistance, and (d) resume monitoring of blast pathogen populations with functional diagnostic markers. International collaboration as well as enhanced linkages within countries are much needed to implement these actions.

Keywords Durable resistance · QTLs · Candidate genes · DNA markers

1 Introduction

Achieving stable resistance to blast is perhaps the most important goal in managing blast disease. Over the past two decades, a shift from an over-reliance on major resistance (R) genes to selection for quantitative resistance has shown success in some breeding programs. In recent years, however, severity of blast disease has increased in Indonesia, Vietnam, the Philippines, South China and South West China. This suggests either an erosion of resistance due to pathogen evolution

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or a lessening of screening efforts in breeding programs, or both. There is an urgency to maintain the stability of blast resistance in these production systems, and to intensify efforts to understand the genetic mechanisms underpinning durable resistance.

Selection for quantitative resistance has been more difficult than selecting for major gene resistance because of its lesser phenotypic effects. Due to epistasis, R genes tend to mask the expression of quantitative resistance. The available whole genome sequence provides the tools for selection of specific genes and reconstitution of a desirable genetic background. It is now technically feasible to combine high-quality blast R (*Pi*) genes with multiple QTL. The challenge is to know what constitutes a high-quality R gene, and what are the important genes or chromosomal combinations that collectively confer durable resistance.

In this paper, we will review what is known about varieties with a reputation for sustained blast resistance in the field and the approaches taken to dissect the resistance in such varieties. We suggest that genetic mapping, saturation mutagenesis, and transcriptome analysis can together provide a genome-wide view of the essential regions for disease resistance, enabling the creation and selection of desirable gene combinations.

2 Phenotyping and Screening Methodologies to Identify Durable Resistance

In general, evaluation of blast resistance after greenhouse inoculation or natural infestations in disease screening nurseries, and field tests are the most common screening methods used in breeding programs. Greenhouse inoculation is suitable for detecting qualitative resistance against specific pathogen isolates whereas blast nursery and field tests are preferred for evaluating quantitative resistance against the native populations. A well-managed blast nursery offers the advantage of screening a large number of lines against diverse pathogen populations. In the 1980s, IRRI implemented a strategy of avoiding extreme resistance phenotypes in screening early generation breeding materials (Marchetti and Bonman 1989). In such a protocol, highly susceptible genotypes are removed but moderately resistant lines are advanced for further testing. This allows for retention of quantitative resistance in the presence of major resistance genes. This strategy, still practiced at IRRI, has been a key for sustaining a good level of quantitative resistance in advanced breeding lines.

Ideally, breeding lines should be tested in multiple locations over several years to ensure adequate exposure to diverse pathogen populations. Extensive field testing, however, is often not possible due to logistics and expenses. Rice researchers in Korea have developed a sequential planting method that enables screening for durable resistance under artificial conditions in a short time. This screening method aims at differentiating the level of resistance of elite breeding lines relative to those varieties with known levels of resistance in the field. Under this procedure, rice

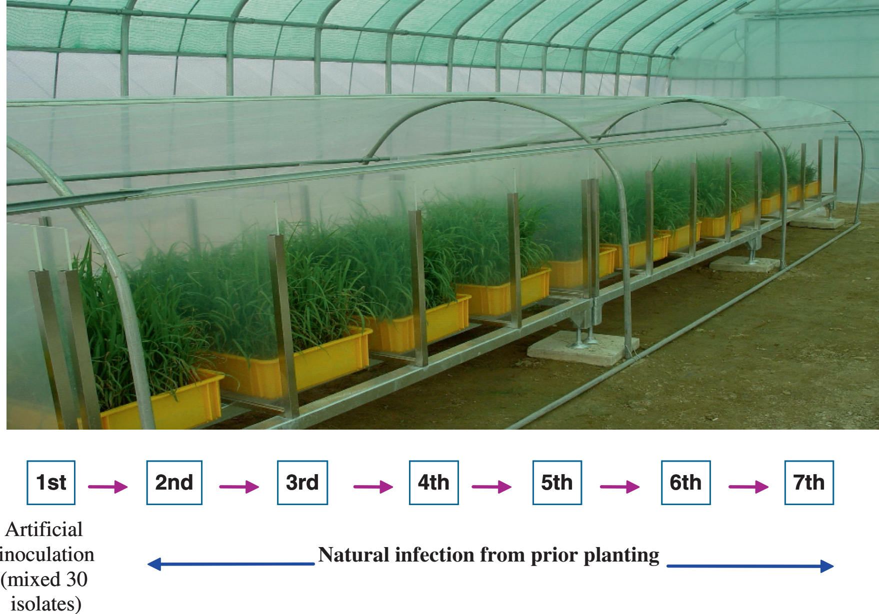

Fig. 1 Sequential planting method to evaluate potential for durable resistance. The screening procedure uses diverse inocula and polycyclic infection to assess the level of resistance in test lines relative to varieties with known level of resistance in the field

lines are planted in rectangular containers ($40 \times 30 \times 10$ cm) at 14-day intervals, with a total of seven overlapping plantings. The first emerged seedlings with 4–5 leaves are given the first round of inoculation. The inoculum consists of a mixture of diverse isolates collected from the field. Seedlings that emerge later are exposed to blast spores generated from the previous inoculation (Fig. 1). The level of disease is estimated by calculating the lesion development rate on each rice line from the first to the 7th planting. Lines showing lesion development rate below 40% are considered worthy for advanced testing. Ranking of lines based on sequential screening appears to correlate with their performance observed in the field. For example, Palgongbyeo, a variety considered to have durable resistance in the field, shows low lesion development in sequential planting experiments.

3 Sources of Durable Blast Resistance

Durable resistance was suggested by Johnson (1981, 1984) to be a retrospective assessment of varieties grown over large area and for a long time. Based on the criteria of time and space, varieties with stable resistance to blast can be identified by examining their performance records in breeding programs. By definition, only varieties widely adopted by farmers can be subject to the durability assessment.

Some varieties may possess durable resistance, yet they have never been planted over large area due to reasons other than blast resistance. Thus, the occurrence of gene combinations conferring durable resistance may be more frequent than that suggested by historical data.

Table 1 summarizes the features of varieties considered to have durable resistance. Most of these varieties had been planted to large acreage for some time. They were replaced not because of loss of blast resistance but due to lesser yield or quality compared to newer varieties. It is instructive to learn how resistance is assessed in some of these varieties, and to understand the genetic mechanisms contributing to the observed stability of blast resistance.

San-Huang-Zhan No 2 (SHZ-2) is an indica rice variety released in 1985 by Guangdong Academy of Agricultural Sciences. Inoculation tests using 340 isolates from eight provinces in China and 50 isolates representing 14 DNA lineages in the Philippines revealed broad-spectrum resistance in SHZ-2 (Zhu et al. 1996; Liu 2001). SHZ-2 expressed resistance to 96% of the isolates from China and 98% of the isolates from the Philippines. Field tests in 74 blast nurseries in different countries also indicated that the resistance of SHZ-2 was better than most entries, including Moreberekan, a landrace variety with a reputation of durable blast resistance (Zhu et al. 2003).

In general, SHZ-2 exhibited low diseased leaf area (DLA), small lesion size and fewer lesions when exposed to diverse and virulent isolates. The high correlations in levels of resistance against diverse pathogen isolates under field conditions suggest that the quantitative resistance to blast is not race-specific and may account for the durable blast resistance observed in SHZ-2. SHZ-2 has been widely used for breeding in South China for more than 20 years and its resistance remains stable. So far, more than 10 varieties derived from SHZ-2 have been developed and the cumulative planting area of these varieties was over 666,000 hectares (Zhu et al. 2003).

Two blast resistant advanced backcross lines, BC-10 and BC-116, were developed using SHZ-2 as donor and Texianzhan 13 (TXZ-13) as recurrent parent (Liu et al. 2004). TXZ-13 is an *indica* variety with high yield, good quality but poor resistance to blast. Field testing in three blast nurseries in Guangdong province for seven consecutive years (14 cropping seasons) showed that the two advanced BC lines exhibited stable leaf and panicle blast resistance. Using BC-10 as restorer line, a blast resistant hybrid variety, Yueza 763, was released in 2007 (Wang et al., unpublished data). In addition to South China, SHZ-2 has been widely used as a blast resistant donor for breeding programs in the Philippines, Indonesia, Iran, India, Korea, and Vietnam.

Gumei 2 is an improved indica rice for irrigated system along the Yangtze River area and is the only semi-dwarf variety possessing durable blast resistance among 38,000 accessions tested during the 1990s (Peng et al. 1996). Gumei 2 has shown consistent resistance in six blast nurseries covering the major ecosystems in China and resistant to 157 pathogen isolates tested in the greenhouse. The variety also showed strong resistance against 40 blast isolates representing 10 common clonal lineages of the pathogen in the Philippines (Wu 2000).

Table 1 Rice varieties with a reputation of possessing durable resistance against blast

Variety	Origin	Varietal type	Genetic attribute and historical performance	Used in breeding programs	Reference
Moroberekan	Africa	Tropical japonica	Multiple R genes, QTL (but may not have been widely planted)	India, Korea	Wang et al. 1994
Palgongbyeo	South Korea	japonica	At least 2 R genes (*Pia, Pib*) Stable resistance for 20 years in blast nursery	Korea	
Owarihatamochi	Japan	japonica	A recessive gene, *pi21*, responsible for stable resistance in the upland rice variety	Japan	Fukuoka and Okuno 2001
Oryzica Llanos 5	Latin America	Indica	? R genes, QTL	Latin America, Indonesia	S. Hulbert, personal communication
San Huang Zhan-2	South China	Indica	3 R genes, 5 QTL > 10 years in China	China, Philippines, Indonesia, Korea, Iran, Vietnam	Liu et al. 2004
Gumei 2	Sichuan, China	Indica	3 R genes, > 9 QTL resistance in blast nursery, and more than 197 isolates tested in greenhouse	China	Wu et al. 2005
IR64	Philippines (tropical and subtropical Asia)	indica	6 R genes; QTL, good for 15–20 years in irrigated lowland	Many countries	Sallaud et al. 2003

IR64, released in 1984, has been widely grown by farmers in tropical Asia. This is attributed to the fact that IR64 possesses many favorable agronomic traits, such as disease and insect resistance, high milling property, and good eating quality. These attributes together make IR64 one of the most popular rice varieties, and grown over 9 million ha at one time. For over 15 years, blast resistance in IR64 was effective in the irrigated rice environments. However, in the early 2000s, signs of gradual erosion of resistance appeared in Indonesia and the Philippines. Currently compatible isolates can be found in the field even though the variety still shows a moderate level of quantitative resistance.

Palgonbyeo, a japonica rice, was released in 1986 in Korea as a high-yielding and good eating quality rice. This variety showed strong resistance both in farmers' field and in blast nurseries. It had been cultivated more than 10 years without severe infection. At one time, Palgongbyeo was planted up to 3% of total rice acreage in Korea, but the acreage gradually decreased due to a shift in consumers' preference to high quality rice. This variety showed stable resistance in 14 different blast screening sites for 20 years. Palgongbyeo, however, is not immune to infection. In greenhouse tests, Palgongbyeo showed susceptibility to 34.7% and 7.1% of Korean and Philippines isolates, respectively.

Moroberekan is a traditional tropical japonica variety grown in Africa. Moroberekan is used as donor of "durable resistance" in the study by Wang et al. (1994), which, for the first time, defined the genetic basis of durable resistance. However, Moroberekan has not been planted to large area. Thus, in a practical sense, there is not enough field data to support the claim that Morobereken is a durably resistant variety.

Oryzica Llanos 5 (OL5), an indica rice, was released as a variety in 1989 in Colombia and resistance breakdown has not been reported for $>$ 15 years. The partial resistance in OL5 to different blast isolates is derived from its parents IR36, Colombia 1, line 5685, Cica 7 and Cica 9 (Correa-Victoria et al. 2004). OL5 shows a stable and high level of partial resistance to blast in Colombia, Indonesia, IRRI, and other blast nurseries around the world. In a recent study, it was reported that blast resistance in OL5 is due to the combined effects of multiple loci with major and minor effects. Some of these mapped to regions of previously identified *Pi* genes but two mapped to regions with no reported *Pi* genes (S. Hulbert, personal communication).

4 Genetic Basis of Durable Resistance

4.1 Right Mix of Genes

Results from genetic analyses of the "presumed" durable resistant varieties suggest that in most cases, this form of resistance is conferred by a combination of major R genes and QTL. In analyzing durable resistance using RIL derived from Moroberekan x Co39, Wang et al. (1994) mapped two R genes (*Pi5* and *Pi7*) and

10 chromosomal regions associated with reduced lesion size. This represents the first experimental evidence of complex genetic control of resistance in a traditional variety exhibiting broad-spectrum resistance. Wu et al. (2004) continued to use Moroberekan as a donor to improve an upland variety Vandana in India. Backcross progeny carrying specific chromosomal regions from Moroberekan appeared to be associated with improved blast resistance in Vandana.

For SHZ-2, strong blast resistance is observed under both natural and artificial inoculations in the Philippines and Guangdong, China. Genetic analyses showed that at least three major genes, tentatively designated as *Pi-GD*-1(t), *Pi-GD*-2(t) and *Pi-GD*-3(t), were responsible for the qualitative resistance to blast in SHZ-2. Five heterologous defense response (DR) genes were significantly associated with DLA in RI lines. Germin-like proteins (previously called oxalate oxidase-like proteins) exhibited the strongest effect, explaining 30.0% of DLA variation. PR-1 and dehydrin also had a significant effect, explaining 15.8 and 23.0% of DLA variation, respectively. Chitinase and the 14-3-3 protein had smaller effects on DLA variation. Together, these five DR markers or genes linked to them contributed 60.3% of the total DLA variation (Liu et al. 2004). QTL analysis using advanced backcross populations suggested that relatively few chromosomal regions (5–10) can confer adequate resistance (Y. Liu et al., unpublished data), indicating that it is feasible to reconstitute the durability of resistance as seen in the donor parent.

Blast resistance in Gumei 2 is controlled by both major and minor genes with epistatic effects. For partial resistance, nine QTLs responsible for percentage DLA, lesion number and lesion size were identified using a RIL population derived from Zhong156/Gumei 2 inoculated with a virulent strain Ca89 in the Philippines. Epistatic interactions both for DLA and lesion number were detected, but not for lesion size. At least three major resistance genes have been identified in Gumei 2, one gene located in chromosome 2 near the *Pib* locus was identified using Philippine isolates. The other two genes, *Pi25(t)* and *P26(t)*, are both located on the short arm of chromosome 6, which appeared to account for the strong resistance observed in screening sites in Fujian province (Wu et al. 1999). However, additional resistance genes including *Pi9*, *Pi2*, Piz^t and *Pigm(t)*, are also known in the vicinity, and allelism of these genes to *Pi25(t)* and *Pi26(t)* has not been determined. It is possible that a cluster of resistance genes in this region contributes to the broad-spectrum blast resistance observed in Gumei 2 (Wu et al. 2005).

4.2 High-Quality Pi Genes

Over 40 *Pi* genes have been named and genetically defined, although their allelic relationships are not all clarified (Table 2). Because *Pi* genes are assumed to function as recognition factors that specifically match pathogen effectors (avirulence factors),

Table 2 Major blast resistance genes (*Pi*) that have been isolated or actively used in breeding programs

Pi-gene	Designation	Donor	Chromosome	Class of gene	Generation of near-isogenic line				Country where being used in breeding	Reference
					Monogenic line	Near-isogenic line				
					LTH	LTH	CO-39	US-2		
Piz	IRBLz-Fu	Fukunishiki ML: IRBLz-Fu	6	–	BC1F20 –	BC3F1 –	– –	– BC4F1		Koizumi, 2007; Kobayashi et al., 2007
Pish	IRBLsh-S	Shin 2 MlL: IRBLsh-S	1	–	BC1F20 –	– BC3F1	BC6F14 BC4F1	– BC3F1	Philippines	Koizumi, 2007; Kobayashi et al., 2007
Pi1	IRBL1-CL (C101LAC)	C101LAC ML:IRBL1-CL (C101LAC)	11	–	BC3F18 –	BC6F16 –	BC6F14 –	– BC6F7	Indonesia; India	Koizumi, 2007; Kobayashi et al., 2007
Pi3	IRBL3-CP4 (C104PKT)	Pai-kan-tao ML: IRBL3-CP4 (C104PKT) Taebeg	9	–	BC2F18 – –	BC6F16 – BC6F15	– BC4F1 –	– BC4F1 –	Vietnam	Koizumi, 2007; Kobayashi et al., 2007
Piz-5 = *Pi2*	IRBLz-CA (C101A51)	C101A51 ML: IRBLz-CA	6	NBS-LRR (*Nbs4-Pi2*)	BC3F18 –	BC6F16 –	BC6F14 –	– BC4F1	Indonesia	Koizumi, 2007; Kobayashi et al., 2007; Zhou et al., 2006
Pi5 (t)	IRBL5-M	RIL249 (Moroberekan) ML:IRBL5-M (RIL249)	9	–	BC3F18 –	BC6F16 –	BC6F14 –	– BC6F8	–	Koizumi, 2007; Kobayashi et al., 2007
Pi9 (t)[a]	IRBL9-W	*Oryza minuta* WHD-IS-75-1-127 ML:IRBL9-W (WHD-IS-75-1-127)	6	NBS-LRR (*Nbs2-Pi9*)	BC3F18 –	BC6F16 –	– BC3F1	– BC6F7	India	Qu et al., 2006

Pii	IRBLi-F5	Fujisaka5 ML:IRBLi-F5 (Fujisaka5)	9?	–	BC1F20 –	– BC3F1	– BC4F1	– BC3F1	–	Koizumi, 2007; Kobayashi et al., 2007
Pik-p	IRBLkp-K60	HR 22 K60 ML:IRBLkp-K60 (K60)	11	–	BC1F20 –	– BC3F1	BC6F14 –	– BC6F7	–	Koizumi, 2007; Kobayashi et al., 2007
Pik-h	IRBLkh-K3	HR 22 K3 ML: IRBLkh-K3 (K3)	11	–	BC1F15 –	BC6F14 –	BC6F14 –	– BC6F7	Bangladesh	Koizumi, 2007; Kobayashi et al., 2007
Pi36	–	Kasalath	8	Rice coiled-coil-NBS-LRR, resembles *Mla1* and *Mla6* than *Pita*, *Pib*, *Pi9*, and *Piz-t*.				None	None; evaluated against Chinese isolates	
Pi40(t)	IR65482-4-136-2-2[b]	*O. australiensis* (Acc. 100882)	6	NBS-LRR motifs	–	–	–	–	–	Jeung et al., 2007

[a] Highly resistant or resistant in polycyclic test at IRRI

[b] Introgression line that inherited the resistance gene from an EE genome wild *Oryza* species, *O. australiensis* (Acc. 100882)

they can be evaded by mutations in the corresponding effectors of the pathogen. High-quality *Pi* genes can be defined as those that are less prone to evasion by the pathogen. In terms of co-evolution, *Pi* genes imposing fitness penalty in mutations of the pathogen are expected to be effective and durable.

Based on inoculation tests with multiple isolates, some R genes appear to provide effective resistance though it is not known whether these genes will eventually meet the criteria of durability after deployment on a large scale. For example, *Pi1* and *Pi2* when used together were thought to confer resistance to many pathogen races and therefore be potentially durable, yet compatible races were found in Indonesia prior to their deployment (Masdiar Bustamam, personal communication). Another R gene *Pi40* has been fined mapped and shown to have broad-spectrum resistance against diverse pathogen populations (Jeung et al. 2007). *Pi40* was originally introgressed from wild rice relative *O. australiensis*. Interestingly, *Pi40* is located on the narrow region of chromosome 6 containing previously mapped *Piz*, *Piz-5*, *Piz-t, Pi9*, and the resistance gene from Gumei 2. Clustering of functionally defined *Pi* genes appears to be common in the rice genome. An interesting question is whether they function individually or collectively in conferring the observed broad-spectrum resistance.

In a rare case, a recessive gene *pi21* was found to confer quantitative blast resistance in the upland variety Owarihatamochi (Fukuoka and Okuno 2001). Preliminary reports suggest that *pi21* is a deletion mutation of an expressed gene of unknown function. The wild type gene encodes a sequence with a heavy metal-enriching domain. Resistance is a result of loss of negative regulation.

So far, field performance has been the main criterion to assess effectiveness of blast resistance. There are no reliable predictors to suggest which genes or gene combinations will be better in large-scale deployment. In bacterial blight, identification and characterization of effectors (avirulence genes) from *Xanthomonas oryzae* pv *oryzae* provides a predictive strategy based on which gene combinations may impose strong fitness penalty on the pathogen (Vera Cruz et al. 2000; Leach et al. 2001; Leung 2008). Adopting a similar strategy would require a better understanding of the effectors in *M. oryzae*. Analysis of the avirulence genes *AVR-Pita* of *M. oryzae* has not revealed obvious parasitic fitness function (Jia et al. 2000). Considerable sequence variation is found in *AVR-Pita* alleles in field isolates; some of which appeared to be mutated by endogenous transposon (*Pot3*) (Zhou et al. 2007). These results suggest a low efficacy of the corresponding R gene, *Pi-ta*. On the other hand, *Pi33* is considered an effective R gene. The corresponding avirulence gene for *Pi33* is called *A*virulence *C*onferring *E*nzyme 1 (*ACE1*) which encodes a polyketide synthase involved in the biosynthesis of secondary metabolites (Bohnert et al. 2004). Whether the effectiveness of *Pi33* is related to the fitness of *ACE1* is not known. Terauchi et al. (2005) described a high-throughput approach of isolating candidate effectors from pathogens. This can potentially lead to the development of a set of molecular markers that are diagnostic for pathogenic functions, providing a guide for the use of *Pi* genes and gene combinations.

4.3 Genes Underlying Disease Resistance QTL

Although many disease resistance QTL have been declared in mapping experiments, the reported intervals are often too large to give breeders confidence to practice selection. Even within a relatively small region, attempts to assign function to genes are complicated by the large number of potential candidates. Furthermore, ascertaining the contribution of candidate defense genes to blast resistance is difficult because these genes often exist as members of gene families. Two approaches have shown promising results towards defining the genetic control of disease resistance QTL. The first involves gene-silencing of single or multiple members of a gene family, and the second involves detection of gene expression signatures that are correlated with phenotypic variation.

Some candidate defense genes, notably members of the gene families of oxalate oxidase (*OsOXO*) and germin-like proteins (*OxGLP*) are associated with blast resistance. Contribution to blast resistance can be demonstrated by silencing of selected defense genes. Analysis of 12 members of the *OxGLP* gene family on chromosome 8 suggested that some members of the gene family contribute resistance not only to blast but also to sheath blight caused by another fungal pathogen, *Rhizoctonia solani* (P. Monosalva et al. personal communication). In this case, the contiguous *OsGLP* genes (80 kb) are contained within the $\sim$ 3.0 Mb complex QTL for quantitative resistance. How common is such a phenomenon? This question can be addressed by examining the whole-genome expression patterns.

Using the 22 K Agilent oligoarrays, we analyzed resistance transcriptomes of a pair of resistant and susceptible germplasm as well as a pair of mutant and wild type lines with contrasting resistance phenotypes (R. Mauleon, K. Satoh, S. Kikuchi et al., unpublished). The results suggest that aggregated and correlated expression patterns can reveal defined chromosomal regions that may contribute to resistance. This raises the interesting possibility that coordinated expression of groups of adjacent genes may function as a "complex QTL". While this is an attractive hypothesis, we are yet unable to show statistically significant alignment between genomic regions showing correlated expression and the blast resistance QTL reported in the literature (Wisser et al. 2005). This could be attributed to the large regions defined by conventional QTL mapping which usually spans over 5–10 cM, covering hundreds of genes. To refine such alignment, a higher resolution of genetic mapping through NIL development is necessary.

5 Saturation Mutagenesis of Genes Important for Blast Resistance

To identify the genomic regions with effects on blast resistance, we screened for loss-of-resistance in IR64 mutants under field conditions. Mutants with gain-of-resistance against multiple pathogens have been found at a low frequency. On the

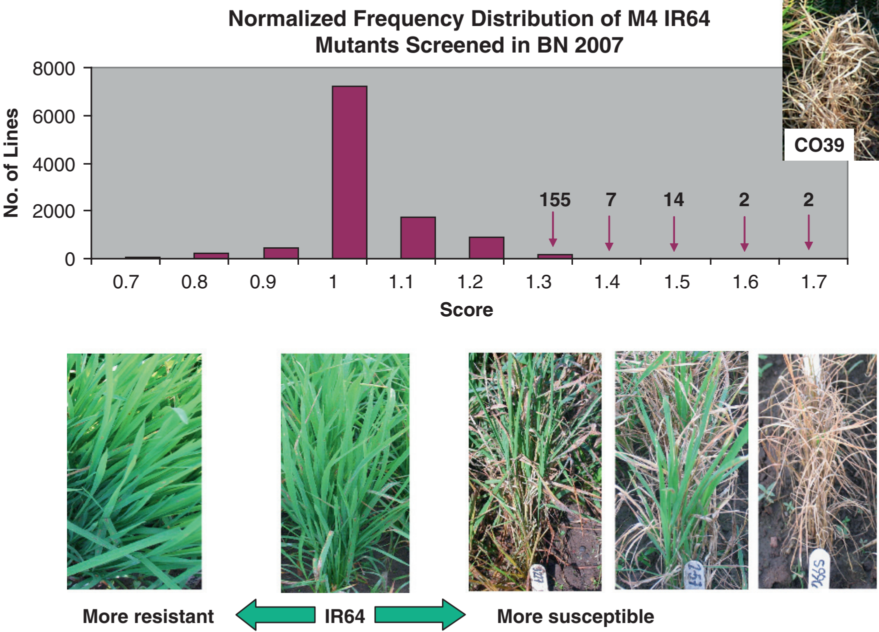

Fig. 2 Screening of IR64 mutants in IRRI blast nursery. Mutant lines with increased or decreased resistance relative to wild type IR64 were recovered for genetic analysis. Screening under polycyclic natural infection in the disease nursery allows an amplification of the disease phenotypes

other hand, loss-of-resistance mutants are common. So far, over 300 susceptible mutants have been recovered from screening > 20, 000 mutant lines (Fig. 2, Table 3). These mutants are important for revealing the genes and genomic regions essential for specific or quantitative resistance to blast.

Relative to IR64, SHZ-2 possesses a stronger level of quantitative resistance. One hypothesis is that SHZ-2 has multiple resistance mechanisms, such that losing one layer of defense mechanism does not lead to complete susceptibility. From screening approximately 7,000 M3 lines of SHZ-2 produced by EMS mutagenesis, we obtained six mutants with enhanced susceptibility. This frequency (0.06%) is about 25-fold lower than that obtained in IR64 (Table 3). Furthermore, SHZ-2 mutants

Table 3 Comparison of frequencies of loss-of-resistance mutations in SHZ-2 and IR64

Genotype	Mutagenesis	Lines screened	Change in susceptibility scores (0–9)	Putative mutants	Percent mutant recovery
SHZ-2	EMS	7, 173	0 to 3	4	0.06
IR64	EMS	2, 000	5.8 to 7.3	31	1.55
	Diepoxybutane	10, 718	4.6 to 5.3	171	1.6
	Gamma ray	12, 691	4.1 to 5	210	1.65
	Fast neutron	4, 289	5.2 to 8.3	33	0.77
All		29, 698	–	445	1.5

Fig. 3 Relative level of susceptibility in SHZ-2 mutants induced by EMS. SHZ-2 mutants show intermediate susceptible phenotypes relative to the severe disease phenotypes observed in IR64

show intermediate rather than highly susceptible phenotypes (Fig. 3). These two observations are consistent with the hypothesis that SHZ has a strong basal level of resistance conferred by multiple loci. Thus, when a single mutation occurs, there is still a substantial level of resistance present that would result in an intermediate resistant phenotype. In other words, there is more "redundancy" in resistance

mechanisms conditioning blast resistance in SHZ-2 than in IR64. Determining the genetic basis of these intermediate disease phenotypes may reveal genes controlling durable resistance.

Based on bioinformatics analysis, Bai et al. (2002) found approximately 600 NB-LRR-like sequences in the rice genome. It is not known which of these have a functional role in disease resistance. It has been shown that genomic deletions in mutants can be detected through hybridization with whole-genome oligoarrays (Gong et al. 2004). Using Affymetrix 57 K RiceChip, we are able to detect putative deletions in 455 gene models using 14 blast susceptible mutants (M. Bruce, G. Diaz, J. Leach et al., unpublished data). We estimated that the median number of detectable gene models is approximately 33 per mutant. With this mutation density, it is estimated that near-saturation deletion mapping can be achieved with 3,000 mutants (with a 92% probability of including a given gene). Systematic analysis of deletion lines showing different degrees of susceptibility may provide a functional scan of genomic regions conferring both qualitative and quantitative resistance against blast.

6 Actions Needed to Sustain Breeding for Durable Resistance Against Blast

Developing durable resistance is a continuous process. Due to the dynamic nature of the pathogen population, erosion of resistance will gradually occur as the pathogen population evolves. It is necessary to have a strategy to anticipate pathogen changes, and to rapidly respond to the threat of the disease. Several steps can be taken to ensure that the genetic resources and knowledge are in place to support an anticipatory breeding approach.

6.1 Develop Breeding-Ready NIL for Pi Genes and QTL

NILs are useful genetic resources for genetic analysis and eventually gene cloning. Currently, there are three sets of NILs developed in three genetic backgrounds: Co39 (indica), LTH (japonica), and US-2 (derived from a cross between Indonesian landrace, Kencana x Takanari 3, an indica type) (Fukuta et al. 2007). These are developed primarily for genetic analysis and for monitoring pathogen populations. The universal susceptibility of Co39 and LTH makes them useful recurrent parents to incorporate *Pi* genes. However, for breeding purpose, the agronomic values of the recurrent parents become important as it determines how widely the NILs will be used in breeding programs. For rapid uptake of NILs in breeding programs, it is necessary to develop NILs containing *Pi* genes and QTL that are suited for breeding. The practical importance of having good recurrent parent is demonstrated in the *Xa* gene NILs for breeding against bacterial blight. The *Xa* NILs are all in IR24 background, a favorable genotype that breeders can immediately use in breeding. With the collection of known *Pi* genes and major QTL found in field-proven varieties,

it is possible to develop NIL series in one or more elite agronomic backgrounds. Marker-aided foreground and background selection can be used to accelerate the NIL development.

6.2 Improve Knowledge of Neck Blast Through Better Screening and Genetic Analysis

Although neck blast (infection at the collar and peduncle tissues) is most damaging to yield and grain quality, relatively little work is done on resistance mechanisms at the reproductive stage. Many screening programs operate with the assumption that seedling resistance will be expressed throughout all plant developmental stages. Is this assumption correct? Screening and analysis of neck blast resistance should be incorporated in key evaluation sites.

6.3 Promote Exchange of Breeding Materials Between Countries for Evaluation and Validation of Durable Resistance

Ability to evaluate genetic materials at multiple sites is essential to assess the spectrum of resistance of breeding lines and gain a glimpse of the potential durability. Performance records from nurseries, such as those organized by the International Network Genetic Evaluation of Rice (INGER), are used as an indicator of durability of resistance in varieties. This will require extensive sharing of genetic materials between countries, perhaps through the facilitation of INGER.

6.4 Resume Monitoring of Blast Pathogen Populations with Functional Diagnostic Markers

Traditionally, pathogen monitoring has been based on DNA fingerprinting with anonymous markers (e.g., genome-wide repetitive DNA sequences), to determine the genetic relationships of pathogen strains and clonal populations. This information is useful in determining the clonal lineage of strains but has limited predictive value on virulence function. With the available genome sequence of the blast fungus, comprehensive profiling of secreted proteins of the fungus is now possible. Some of these proteins may confer effector functions. It is possible to develop a panel of effector molecules as a diagnostic tool for determining the virulence/avirulence spectrum of a given pathogen population, thereby enabling the monitoring of pathogen changes in breeding nurseries and pathogen movement between regions.

7 Conclusion

Compared to a decade ago, we are more efficient in determining the genes or chromosomal regions conferring blast resistance. To translate this knowledge to breeding, specific genetic materials and tools need to be developed to enable

efficient selection. First, we need to develop and maintain well-characterized pre-breeding lines to enable rapid gene pyramiding. The relevance of this pre-breeding gene pool should be based on knowledge of the pathogen populations at different geographical locations. It will require a simple and robust system to track pathogen changes. Second, we need multiple screening sites to validate the effectiveness of the 'reconstituted resistance". It means that we need more efficient germplasm exchange between partners. Third, we need integration of mapping/mutant datasets and collaboration among breeders, pathologists and geneticists across institutions such that results on genetic materials are archived and shared. These activities cannot be done by a single research organization but require collaboration through a network of research and breeding programs.

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Part V
Bioinformatics and Database

Magnaporthe grisea Oryza sativa (MGOS) Interaction Database: Community Annotation

Kevin A. Greer and Carol Soderlund

Abstract The *Magnaporthe grisea Oryza sativa* interaction database (www.mgosdb.org) is a web-based repository for *Oryza sativa* and *Magnaporthe oryzae* genomic and experimental data with a particular emphasis on host-pathogen interactions. MGOS has recently been enhance to include community annotation and enhanced microarray functionality. The new community annotation functionality allows *M. oryzae* researchers to document the *M. Oryzae* transcriptome including: gene name/symbol and description, associated gene ontology and fungal anatomy terms, mutants and associated images, related publications, and transcript information including exon positions and translational start and stop positions. The enhanced microarray functionality allows users to enter all of the data associated with a microarray experiment that is necessary to interpret the results of the experiment. This includes the raw data files for each hybridization, the final processed data set for all hybridizations, the essential sample information (in particular the experimental factors and their values), the experimental design and hybridization scheme, and protocol information. In addition, a community forum system and a literature citation section have been added to the MGOS website.

Keywords MGOS · bioinformatics · *Magnaporthe grisea* · ESTs · SAGE · Gene annotation

1 Introduction

The *Magnaporthe grisea Oryza sativa* interaction database was initially developed as part of a five year National Science Foundation Plant Genome Research Program focused on understanding the basis of plant resistance through a comprehensive analysis of the molecular events that occur during pathogen-host recognition and

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subsequent defense responses. In support of that goal, the MGOS database was developed as a web repository for both pathogen and host genomic and experimental data (Soderlund et al. 2006), and as a portal linking this data with the plethora of information available on the internet. In the fall of 2006 a new grant was awarded to enhance the MGOS database to support community based gene annotation and expand the current microarray functionality to allow web-based submission of microarray and other relevant rice and rice blast data. Over the course of the last year significant progress has been made in the development and implementation of the database and web pages necessary to accomplish these objectives. In addition, new functionality such as a discussion forum and web-based mutant submission has been added at the request of the *Magnaporthe oryzae* research community. Wherever possible, existing tools such as GBrowse (Stein et al. 2002), BioPerl (Stajich et al. 2002), and a local installation of the Gene Ontology Database (Ashburner et al. 2000) were utilized to speed development and improve interoperability with other systems.

2 User Interface and Navigation Enhancements

In order to provide a simpler, more consistent user interface, the MGOS home page was redesigned and a set of templates were designed and applied to all existing web pages. All navigational links, except the home page and site map links, use a ‘breadcrumb’ at the top of the each page (Fig. 1), which provides a history of

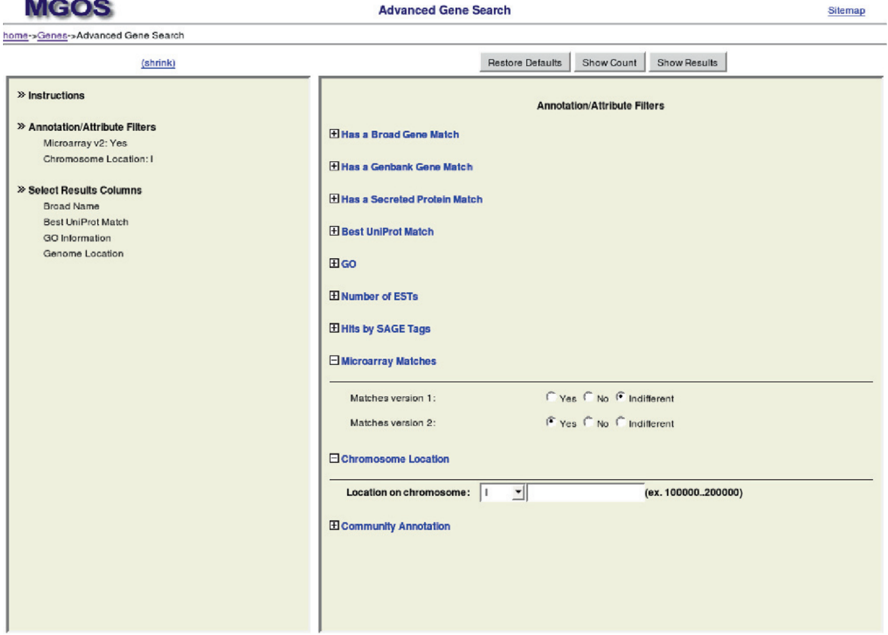

Fig. 1 Advanced gene search displaying new BioMart style interface

the most recent pages that were visited during the current session. The breadcrumb also maintains the state of each page, allowing the user to re-enter a page without loosing the information that had been previously entered on the page. This feature is particularly useful on query pages in that it allows users to return to, and modify previous queries, or return to a result set in the state in which it was left. The page templates employ cascading style sheets to provide a consistent look and feel.

3 Updated Data

Although the experimental data in MGOS such as SAGE, EST libraries, microarray, etc. remains relatively constant, as once it has been entered it is only usually modified to correct an error or omission, the genome and annotation information is still in a state of relative flux. In August 2007 MGOS was updated to include release 5 of the TIGR rice sequenced chromosomes (Yuan et al. 2003, 2005), the latest set of clones from the KOME rice full length cDNA project (Kikuchi et al. 2003), and release 17.0 TIGR rice gene index (Quackenbush et al. 2000).

The *M. oryzae* gene set, which had already been updated based on the Broad version 5 release, was amended using all non-hypothetical *M. oryzae* proteins available in the NCBI Entrez protein database (Wheeler et al. 2007). All *M. oryzae* gene sequences were blasted against an updated list of non-hypothetical fungal proteins downloaded from UniProt (Apweiler et al. 2004) and the gene descriptions were updated for suitable matches. Additional data was also added to MGOS in November, 2007 based on requests received at the 4th International Rice Blast Conference. GenBank accession numbers for all *M. oryzae* genes were downloaded from the NCBI gene database and assigned to MGOS genes. These identifiers are often used in addition to the Broad and MGOS identifiers already present in MGOS. Protein domain information was also determined for the *M. oryzae* genes using the Pfam database (Finn et al. 2006) portion of InterPro (Mulder & Apweiler 2007).

4 Biomart Style Query Interface

The latest release of MGOS includes new advanced querying capabilities, which are accessed through a BioMart (Kasprzyk et al. 2004) style interface. In addition to providing a consistent layout between the advanced query screens, there are common sections available for all queries. As illustrated in Fig. 1, clicking any of the bold links in the left panel, which are preceded by ‘>>’, will result in the corresponding section being displayed in the right panel.

Clicking on the >> **Instructions** link in the left panel will display on the right panel a description of the screen and the procedure for building and executing the query. The >> **Annotations/Attribute Filters** link will display on the right panel the list of attributes that can be used to limit the results returned by the query. Clicking the plus sign that is next to a filter in the right panel will show the options that are used to define the filter criteria. For example, the options selected in Fig. 1 would

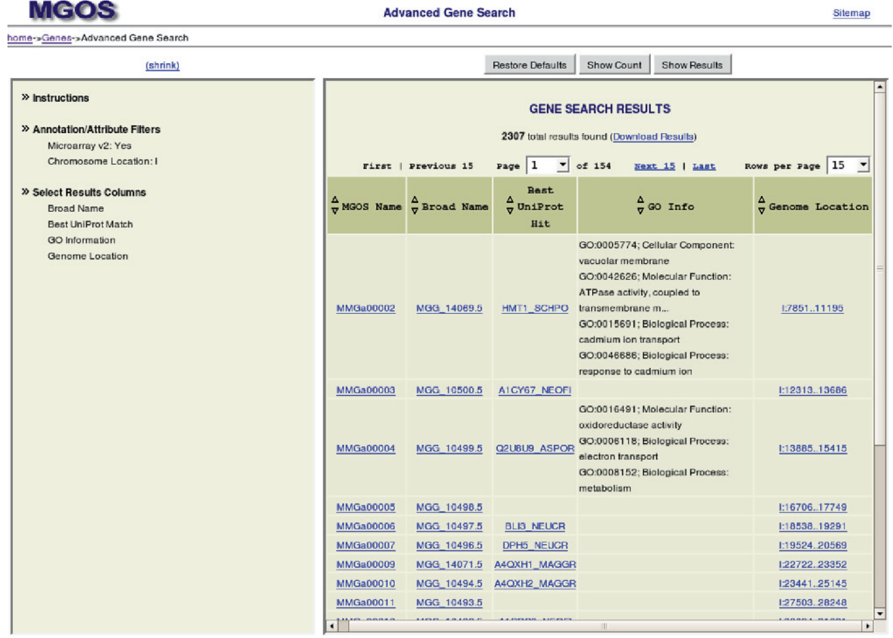

Fig. 2 Advanced gene search query results rendered using tableizer module

result in displaying all genes that have microarray matches and are on chromosome 1. The >> **Select Results Columns** link will display on the right panel the list of columns which are available for inclusion in the result set, which are selected using the checkboxes that are next to each column description. The 'Show Results' button above the right panel will execute the query and display the results using a perl package called 'tableizer' that was developed during the first phase of MGOS development (Soderlund et al. 2006). A 'shrink/expand' link above the left panel has been added in order to allow the left panel to be shrunken, therefore maximizing the amount of space available for viewing results in the right panel (see Fig. 2). This interface renders the results in a table that includes paging, with a user selectable number of rows per page, and the ability to sort on any column (Fig. 2). Additional unique query criteria necessitated by some datasets is implemented by adding links to the left hand panel, thus maintaining a consistent user experience and minimizing the time required to learn how to use each screen.

5 Community Annotation

The most significant enhancement to the MGOS website has been the addition of community annotation functionality. Modeled after the *Neurospora* functional genomics project (Dunlap et al. 2007), the implementation of community annotations

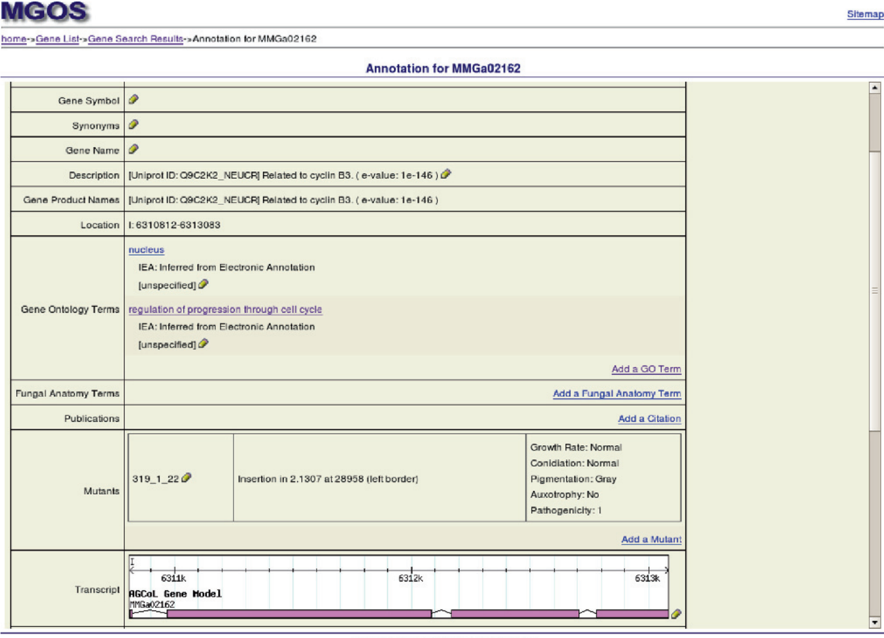

Fig. 3 Community annotation main screen

was designed to allow the *Magnaporthe oryzae* research community to maintain the annotation information for each gene online, including gene name, gene location, gene symbol, synonyms, gene description, associated gene ontology and fungal anatomy terms, related publications, and transcript information including exon positions, translational start and stop positions, and a description of the gene product (Fig. 3). In addition, the ability to maintain and associate mutant information (including image files of the mutants) with an annotation was added based on feedback from the *M. oryzae* community.

Particular attention was paid to the process of transcript entry/maintenance. Specifically, entering a transcript on the negative strand can be somewhat confusing. Consequently, text boxes were added to allow the user to enter the position of each exon relative to the start of the transcript, rather than the chromosomal position, thus avoiding the problems associated with having to calculate the position of each exon on the positive strand (Fig. 4). In addition, a sequence alignment screen has been linked to the transcript maintenance screen that allows the user to paste in his/her sequence and align it to the genome using BLAT(Kent 2002). After reviewing the results of the alignment, the user may retrieve the coordinates from an alignment back into the transcript maintenance screen by clicking the 'Blat a Transcript Sequence' link next to the desired alignment.

Automatic validation of each transcript includes: exons must be non-overlapping, the transcript must have a valid start codon (if the start of the transcript is included in

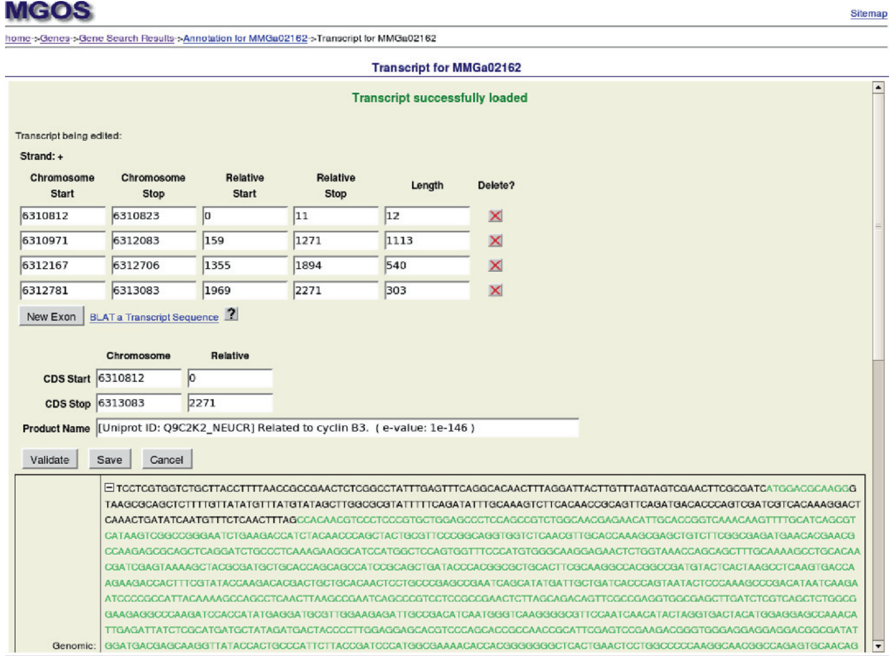

Fig. 4 Community Annotation transcript maintenance screen

the sequence); the transcript must have a valid stop codon (if the end of the transcript is included in the sequence); the coding length must be evenly divisible by three; and a transcript must contain at least on exon. To facilitate the visual validation of the transcript, three different views of the sequence are displayed at the bottom of the transcript maintenance screen, including: the genetic sequence with exons highlighted; the transcript sequence with the coding region highlighted; the corresponding amino acid sequence for the transcript. The main gene annotation page also includes a genome browser (Stein et al. 2002) display of all other features in MGOS that overlap with the genomic coordinates of the gene, as well as links to a java version of the gbrowse viewer (www.agcol.arizona.edu/software/java_gbrowse), and links for performing either local or NCBI blasts of the genomic or amino acid sequences of the gene.

In addition to transcript maintenance, another primary area of focus for community annotations was the association of ontology terms with genes. Currently there are two ontologies available in MGOS, gene ontology (GO) (Ashburner et al. 2000) and fungal anatomy ontology (http://www.yeastgenome.org/fungi/fungal_anatomy_ontology/). These two ontologies were implemented using the same generalized codes, thus allowing additional ontologies to be added with minimal effort. To associate an ontology term with a gene the user simply selects the term, enters the supporting PubMed ID or publication information (if available), and chooses the appropriate evidence code (see Fig. 5).

MGOS Sitemap

home->Mutant Assays->Genes->Advanced Gene Search->Mutant Assay Items for Enzymatic activity->AGCoL Gene Details->Annotation for MMGa02162->Gene Ontology Term for MMGa02162

Gene Ontology Term for MMGa02162

Usage note: If you use the PubMed Id field, the citation field will be populated automatically. To reference a publication that is not in PubMed, leave the PubMed Id field blank.

Gene Ontology Term: Choose Term Gene Ontology Website

Pubmed Id: Open Entrez PubMed

* Authors: [unspecified]

* Title: [unspecified]

* Publication Date: Jan 2008

Journal:

Volume: Issue: Pages:

* Evidence Code: Guide to GO Evidence Codes

Save Cancel

INSTRUCTIONS:
1. Click "Choose Term" to select an ontology term.
2. Enter PubID and press return key -- the citation will be added.
(or type in the citation).
3. Select the most appropriate evidence code.
4. Click the Save button.

Last modified: Tue Dec 11 13:52:56 2007

Fig. 5 Gene Ontology term association screen

6 Literature

As part of the community annotation implementation citation information can be added as support for a genes' annotation. For example, a journal article may be cited in order to support the assignment of a GO term or the determination of the exon boundaries of a transcript. This citation functionality was extended to allow citations to be added to MGOS without being associated with an annotation. In addition a literature screen (Fig. 6) was developed to allow the citations within MGOS to be filtered based on the author, title, and publication date. These filters may be used in combination, and the author and title filter boxes will return results based on a partial match. This screen is used both for browsing the literature, and once the user as logged in (see Forum), to add a citation or select a citation for editing. Citation maintenance is performed using the same screens that were developed for community annotations, and can contain citations that are, or are not, listed in PubMed. To that end, MGOS can serve as a primary repository for all *M. oryzae* related publication citations.

7 Forum

During discussions at the 2007 Plant and Animal Genome conference, a request was made to have a forum added to the MGOS website to facilitate online communication and collaboration. In response to this request, the Simple Machines Forum (SMF) software package (http://www.simplemachines.org) was added to the MGOS

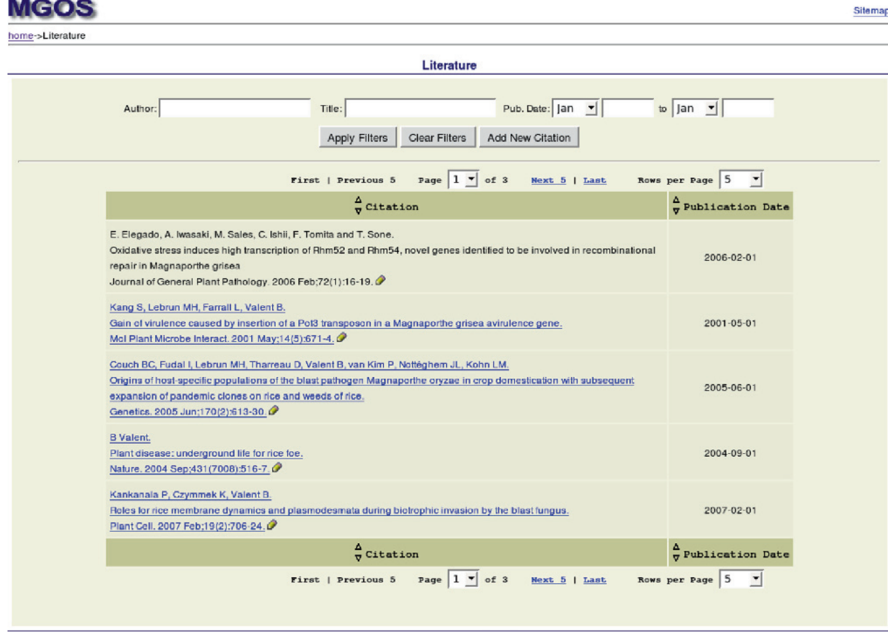

Fig. 6 Literature main screen

web server and configured for the rice blast community. This package was selected based on its functionality, compatible architecture consisting of PHP and MySQL, its cost (free), and its active user and developer communities. In addition to providing moderated online discussion functionality, SMF provides private messaging, a community calendar, and the ability to announce a new topic to all members though email. SMF also provides robust user management functionality which was leveraged to provide the secure user login to MGOS, thus avoiding the need to log-in multiple times, and saving development time. Any member of the forum may add annotations, literature and microarray results to MGOS using their forum user ID and password.

8 Microarray

The Microarray functionality in MGOS has been enhanced to allow end users to upload their experimental datasets directly into MGOS using a web-based interface. During this process the user must provide the basic information described in the MIAME 2.0 proposal (Brazma et al. 2001). During the implementation of this standard, the focus was on gathering the necessary information to interpret the results of a microarray experiment, not requiring all of the information necessary to repeat an experiment (which could be obtained from the literature). To this end, the user

is required to provide the raw data files for each hybridization, the final processed data set for all hybridizations, the essential sample information (in particular the experimental factors and their values), the experimental design and hybridization scheme, and a reference to, or a basic description of the experimental protocols. The user must also select the microarray that was used in the experiment, however the annotation information for each type of microarray will be maintained by the MGOS support staff. Users are also encouraged to upload the hybridization image files and any other supporting files to the MGOS website, however it is not a requirement to do so.

One of the highest priorities of this enhancement to the microarray functionality in MGOS was to provide a way to make microarray data publicly available though a process which is intuitive and expeditious. Information such as protocols, once entered are stored for later use, and a summary of the micoarray experiment is available on one screen that provides links to each section of the experiment. In addition, the system is designed to export data to other public repositories such as the Gene Expression Omnibus (GEO)(Barrett et al. 2007) and ArrayExpress (Parkinson et al. 2007).

9 Future Releases

New releases of the MGOS website will be deployed approximately every three months. The next major release will include a redesigned database necessary to fully support multiple annotations per gene and complete the implementation of the enhanced microarray functionality. The redesigned database will also support multiple species, which will allow genomic sequence and annotation information (with or without corresponding genomic sequence) to be entered for additional *M. oryzae* species. We are also in the process of implementing community annotation related functionality that was specifically requested by the *M. oryzae* research community and welcome suggestions for changes or enhancement that will facility future research.

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ARCHIPELAGO: Towards Bridging the Gap Between Molecular and Genetic Information in Rice Blast Disease Resistance

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Abstract Large amounts of major resistance genes, QTL (Quantitative Trait Locus) and expression data dealing with rice blast resistance have been described in the past. We extensively reviewed this information and inserted it into the OryGenesDB database, creating the ARCHIPELAGO resource. This represents more than 1,500 genes differentially expressed upon infection by *Magnaporthe oryzae*, 1,000 resistance gene analogs, 88 mapped resistance genes, 341 QTLs and 165 metaQTLs. Further analysis of the metaQTL data allowed the identification of robust or broad spectrum metaQTLs. Cross referencing defense gene expression and metaQTL information identified some candidate genes for metaQTL but does not demonstrate preferential co-localization of defense-related genes in metaQTLs. In contrast, disease regulators are statistically associated with disease QTLs. At the genomic level, we can observe that some regions of the rice genome are richer than others for defense-related genes. This resource opens new possibilities for marker-assisted selection and QTL cloning.

Keywords Database · Defense-related genes · Intracellular disease regulators · metaQTL · Resistance genes

1 Introduction

Rice is the staple food for more than half of the world population (Kush 2005). Fungal diseases, especially blast disease caused by *Magnaporthe oryzae*, are a major threat to this crop and can lead to up to 35% loss for a single country like China. Intensive efforts have been made in the past to identify major resistance

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genes as well as Quantitative Trait Loci (QTL) to blast disease. On the other hand, rice has emerged as a model species for Monocots and was fully sequenced (Bennetzen 2002). Thus, rice and rice blast represents one of the first opportunities to integrate information from breeders and genomic information from fundamental studies.

Studies of the past 20 years in the field of plant-pathogen interactions revealed that resistance requires transcriptional activity and can be divided into three steps (Dangl and Jones 2001). In a first step, the presence of the pathogen is detected by the plant throughout different recognition systems involving resistance (R) genes or other pattern recognition receptors (Jones and Dangl 2006). In a second step, signal transduction occurs and requires regulators such as MAP kinases (Mishra et al. 2006) and transcription factors (Eulgem 2005) that can be collectively coined IDR (Intracellular Disease Regulator) genes. In a third step, defense responses are induced. These include production of antimicrobial secondary metabolites (phytoalexins) (Peters 2006), pathogenesis-related (PR) proteins (e.g. chitinases, glucanases) (Jwa et al. 2006), cell-wall strengthening (Hückelhoven 2007) and programmed cell death, leading to the Hypersensitive Response (HR) (Greenberg and Yao 2004). Most genes involved in these three steps are differentially expressed upon infection. In the past 5 years, there has been an exponential increase of information on the rice genes and proteins that are differentially expressed upon infection by pathogens and pathogen-related stresses. As this type of data is still growing, there is clearly the need for an integrated resource that would allow new data sets to be compared with previous results.

Besides this molecular information, the rice community has generated an enormous amount of genetic information on blast disease resistance (*Pi* major resistance genes and blast Quantitative Trait Loci, or QTLs). However, this information remains unlinked to the wealth of molecular information available, in particular the rice genome sequence. Some of the rice disease resistance QTLs to various rice diseases were recently physically anchored to the rice genome (Wisser et al. 2005). This lead to the identification of chromosomal segments associated with quantitative broad-spectrum resistance to blast and blight diseases. Wisser and colleagues also observed that some gene families like RGAs (Resistance Gene Aanalogs), glutathione-S transferases and some proteases were significantly associated with disease QTLs (Wisser et al. 2005). However, this work did not include all available or recent informations.

We reviewed all available expression (Vergne et al. 2008) and genetic studies (Ballini et al. 2008) on blast disease resistance and integrated the resulting resource, called ARCHIPELAGO, into the web-based database OryGenesDB (Droc et al. 2006). This allowed us to bridge the gap between molecular and genetic information on blast disease. Using this resource, here we address the fundamental question of the link between mapped R genes, RGAs, IDR genes, defense-related genes and QTL. Furthermore, we provide the first integrated physical map of the rice defense system, highlighting some QTLs and rice genomic regions that may be useful for breeding for blast resistance.

2 Building the ARCHIPELAGO Resourse

The building of the ARCHIPELAGO resource is described well in Vergne et al. (2008) and Ballini et al. (2008) and will only be briefly summarized here. Two kinds of informations were included: genetic information (mapped *Pi* genes, blast QTL and metaQTL) and structural/functional information (RGAs, IDR genes and genes differentially expressed upon infection by different pathogens or defense-related genes) (Table 1). Altogether, these represent more than 1,000 RGAs and more than 2,500 defense-related genes that are differentially expressed upon infection; most of these genes (around 1,600) are differentially expressed upon *M. oryzae* infection. The genetic information gathered for 88 mapped *Pi* genes, 341 blast QTLs and corresponding 165 metaQTLs (Ballini et al. 2008) was physically anchored to the rice genome on the OrygenesDB (Droc et al. 2006). A genomic view of these data is presented in Fig. 1. From this picture, it appears that some chromosomes are richer than other for defense-related genes (e.g. chromosome 3 versus chromosome 12), in mapped *Pi* genes (e.g. chromosome 12 versus chromosome 3) and in IDR genes (e.g. top of chromosome 2). Finally, defense-related genes are not homogeneously distributed and display hot spots (e.g. top of chromosome 12). This lead us to conclude that the rice defense system is organized with an island-like structure and consequently called this resource ARCHIPELAGO (Vergne et al. 2008).

Table 1 ARCHIPELAGO contents

Type of information	Category	number
genetic information anchored on the genome	Mapped *Pi* genes	88
	QTLs	341
	MetaQTLs	165
structural/functional information	Resistance Gene Analogs	1, 045
	Intracellular Disease regulator	36
	Defense-related genes	2, 562

3 Co-localization of Blast Meta QTLs with RGAs and IDR Genes but not with Defense-Related Genes

Using the ARCHIPELAGO resource, next we addressed the possible co-localizations between *Pi* genes or blast metaQTLs with RGAs, IDR genes and defense-related genes. As previously described (Wisser et al. 2005), RGAs co-localized with mapped *Pi* genes in 59% of the cases (Fig. 2A), significantly more ($P < 0.01$) than with a random set of 2,000 genes. Thus, as expected, RGAs are likely the category of genes behind the *Pi* genes. When tested, 61% of the RGAs

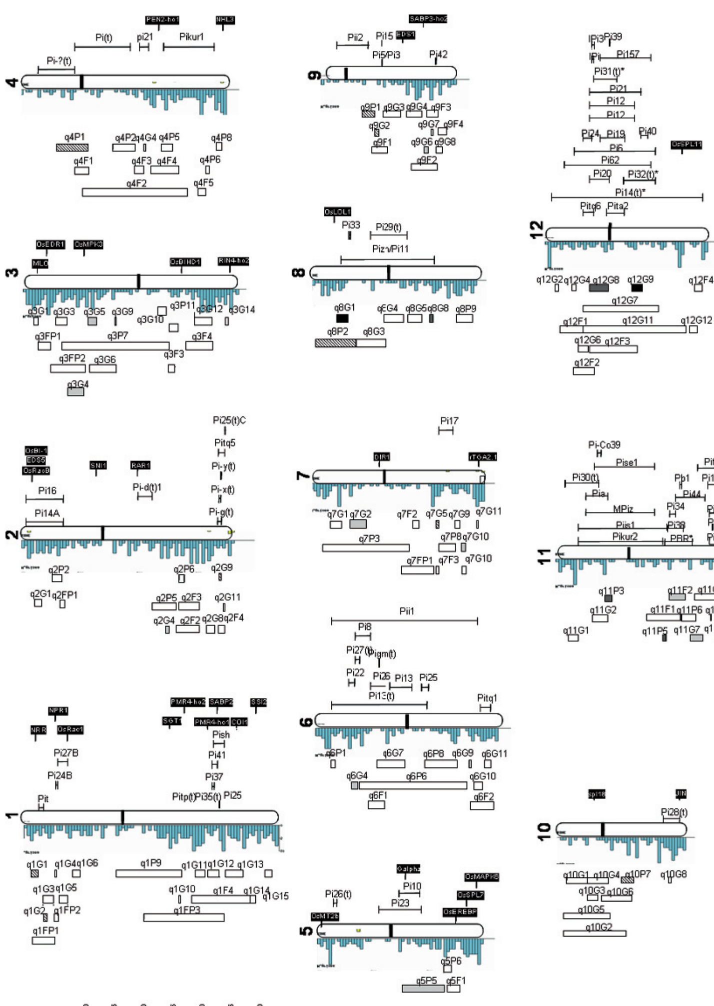

Fig. 1 Overview of blast *Pi*-genes, metaQTL and defense-related genes. MetaQTL are indicated on the *left* of the chromosomes. The colour of the *bars* on the *left* indicates the numbers of studies where the metaQTL was found: three times (*grey*), four times (*dark grey*) and seven times (*black*). Hatched *bars* represent broad-spectrum resistance metaQTL. See Table 2 and text for more details. Density (genes/500 kb) of defense-related genes is indicated by histograms. Genetic position of major resistance *Pi* genes is indicated by *lines* on the *right* of each chromosome. Physical position of IDR genes is indicated by *black bars* with *white labels* on the *right* of each chromosome

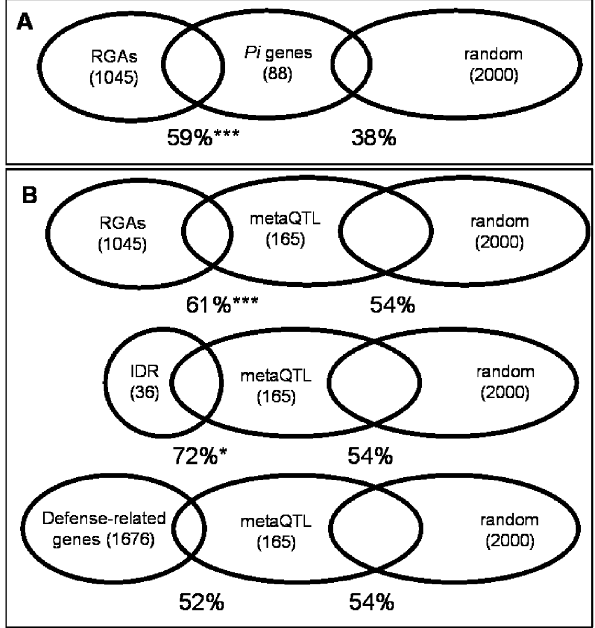

Fig. 2 Co-localizations between *Pi* genes, metaQTLs and defense-related genes. For each category tested (*Pi* genes or metaQTL), possible co-localization of different classes of genes (RGAs: Resistance Gene Analogs; IDR: intracellular Disease Regulator; Defense-related genes: genes differentially expressed upon infection by *M. oryzae*) were tested. The percentage of items showing co-localization is indicated. A control set of 2000 randomly selected genes was used to establish if co-localizations were likely or not found by chance (t test, bonferroni correction: * $P < 0.05$, *** $P < 0.01$). Numbers between parenthesis represent the number of items in each category

also co-localized with blast metaQTLs (Fig. 2B). This suggests that RGAs are likely responsible for most of the metaQTLs identified. We also observed that 72% of the IDR genes abnormally co-localized ($P < 0.05$) with metaQTLs, suggesting that these genes could also explain some metaQTLs. Finally, we did not detect a significant enrichment of metaQTLs with defense-related genes differentially expressed upon *M. oryzae* infection.

4 Identification of the Most Robust and Broad Spectrum Metaqtl for Blast Resistance

Using ARCHIPELAGO, we could ask what were the QTLs, and consequently the metaQTLs, that were found in several independent studies. These metaQTLs represent regions of the rice genome that can be considered as robust in terms of reproducibility. We could find 11 metaQTLs identified in four studies, three in five studies and two in seven studies (Table 2). Thus there are 16 metaQTLs that are identified most of the time in quantitative analysis of blast resistance. These robust metaQTLs explain 23% of resistance and are effective against 17% of the isolates tested on average (seven isolates on average). In order to identify metaQTLs that

Table 2 Most robust and broad-spectrum metaQTL for blast disease resistance BSR: broad-spectrum resistance

metaQTL Name	Class	Number of independent studies	LOD max	R2 max	Number of isolates tested	Number of isolates recognized	Percent isolate recognzed	Donor Varities	Chr	size (Mb)	Closest flanking markers	
q8P2	BSR	1	16.7	46.5	18	15	83	Bala	8	7.129	RM22189	RM22628
q9P1	BSR	1	13.0	21.5	18	6	33	Azucena	9	2.33	R1164	R1687
q9G2	BSR	1	> 2.4	18	15	14	93	CT 9993-5-10-M	9	0.87	R1687	RG553
q10P7	BSR	1	7.6	15.4	18	8	44	Azucena	10	2.44	RG257	G1082
q1G1	BSR	2	9.0	17	21	7	33	Minghui 63, ZYQ8	1	1.29	RM3148	RM10111
q2G9	BSR	2	27.8	25.5	21	10	48	Minghui 63, ZYQ8	2	0.34	RM14154	RM14178
q7G5	BSR	2	5.0	12	33	17	52	CT 9993-5-10-M, Azucena	7	0.31	RM336	RM21822
q1G2	BSR	3	11.9	25.9	23	7	30	Zhong 156, ZYQ8, Moroberekan	1	0.78	RM10145	RM1321
q4P1	BSR	3	9.0	20.2	29	11	38	Lemont, Bala, Azucena	4	5.64	RM16460	RM16584
q2G4	robust	4	5.3	14.7	48	4	8	JX17, Bala, Oryzica Llanos 5, Gumei 2	2	0.71	RM13626	RM1307
q3G4	robust	4	5.1	9.29	43	4	9	Gumei 2, Bala, ZYQ8, Azucena	3	2.91	RM3716	RM14760
q3G5	robust	4	5.2	27.8	38	8	21	ZYQ8, Teqing, Fanny, Bala	3	1.67	RM14798	RM14886
q5P5	robust	4	6.1	24	32	7	22	Azucena, Lemont, Moroberekan, Azucena	5	8.13	RM18408	RM18882
q6G4	robust	4	5.9	16	45	6	13	Azucena, Bala, JX17	6	1.22	RM19574	RM19665
q6G9	robust	4	4.9	14.3	44	7	16	Bala, JX17, Azucena	6	0.33	RM2049	RM20508
q7G2	robust	4	5.1	11.1	32	5	16	Teqing, Bala, Azucena	7	2.92	RM7161	RM21328
q7G10	robust	4	6.3	17.8	42	6	14	ZYQ8, Bala	7	0.78	RM22041	RM22068
q9G6	robust	4	7.5	12.2	30	7	23	Zhenshan 97, ZYQ8, Oryzica Llanos 5, Azucena	9	0.91	RM3533	RM242
q11F2	robust	4	29.7	45.6	*Field*	*Field*	*nd*	Chubu 32, Moroberekan, Lemont	11	3.15	RM26869	RM27030
q11G7	robust	4	7.0	12.1	50	6	12	Gumei 2, ZYQ8, Bala, Lemont	11	2.45	RM27057	RM27222
q8G8	robust	5	7.2	15.6	59	10	17	Bala, ZYQ8, Lemont, Fanny	8	0.53	RM23132	RM23170
q11G5	robust	5	8.8	24	39	5	13	Moroberekan, Azucena, Oryzica Llanos 5, Teqing, Gumei 2	11	0.51	RM26816	RM26840
q12G8	robust	5	19.4	54	36	10	28	Oryzica Llanos 5, IR64, Azucena	12	3.63	RM27785	RM27936
q8G1	robust	7	12.0	32.9	53	12	23	ZYQ8, Gumei 2, Oryzica Llanos 5, Bala, Moroberekan, Minghui 63	8	1.96	RM22453	RM6208
q12G9	robust	7	20.8	32.7	57	14	25	Moroberekan, Azucena, Bala, Oryzica Llanos 5, ZYQ8	12	1.84	RM28060	RM28127

would confer broad-spectrum resistance (BSR), we selected those which were effective against more than 30% of the isolates tested (more than five isolates tested). This represents nine BSR metaQTLs (Table 2) that explain 22% of resistance and are effective against 51% of the isolates tested on average (11 isolates on average).

Robust and BSR metaQTLs could be found in both indica and japonica rice subgroups and some were found in up to six rice genotypes (e.g. q8G1). Most proximal microsatellite markers were identified for all robust and BSR metaQTLs as a first step towards using this information for marker-assisted selection (Table 2).

5 Identification of Candidate Genes for Blast R-Genes and Meta QTLs

Considering that RGAs were good candidates for mapped *Pi* genes (Fig. 2A), we looked for *Pi* genes that were found in small physical intervals to see if candidates RGAs could be identified. We identified seven *Pi* loci smaller than 500 kb and for which they were only one or two RGAs. Thus these RGAs could be candidates for the corresponding *Pi* genes. However, due to background differences, these genes may not be good candidates.

Candidate gene approach appears difficult for most of 80% of the metaQTLs that are larger than 500 kb. Only a small number of metaQTLs (17) represents genomic regions smaller than 500 kb. Although there is no global co-localization of defense-related genes and metaQTLs (Fig. 2B), it is still possible, in some cases, that defense-related genes are responsible of some QTLs. We could find four QTLs smaller than 500 kb in which defense-related gene density is high and where there is no RGA. Interestingly, one of these QTLs (Fig. 3) contains two endochitinases, one of which belongs to the few genes under the control of *CeBIP* receptor for chitin (Kaku et al. 2006).

6 Concluding Remarks

Two major models for quantitative resistance are usually proposed. In the first model, quantitative resistance is the result of defective R genes that can recognize pathogens with low efficiency. This is supported by the observation that QTLs, R genes and resistance gene analogs (RGAs) seem to co-localize (Wisser et al. 2005). A second model, which may not be exclusive, suggests that defense-related genes, as the final executers of resistance, explain significant parts of quantitative resistance. This is supported by the works of Ramalingam et al. (2003) and Liu et al. (2004) that show that marker-assisted selection using defense-related genes improves blast resistance in rice. However, there is no global demonstration of statistically significant co-localization of defense-related genes and QTL. Here, we show that RGAs can explain metaQTLs but defense-related genes are not massively associated with

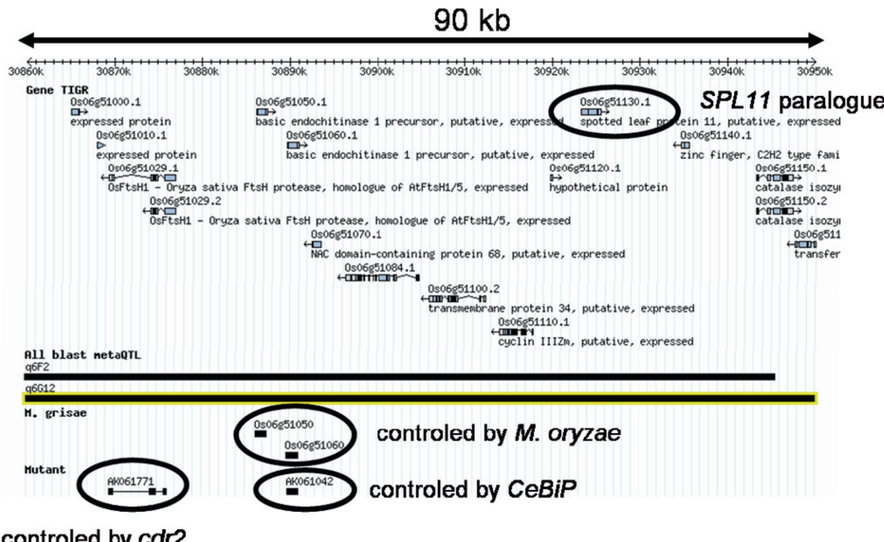

Fig. 3 An example of small metaQTL. This metaQTL contains two defense-related genes (basic endochitinases) induced by *M. oryzae*, one of which is controled by the IDR gene *CeBIP*, one gene induced in the lesion mimic mutant cdr2 and one paralogue of SPL11, a lesion mimic gene

metaQTLs (Fig. 2B). Thus breeders should be careful in using the latter class of genes to marker-assist selection for blast disease resistance.

An alternative model for metaQTLs which has not been thoroughly explored is the possibility that IDR genes could explain some QTLs. Here we show that these IDR genes in some cases (e.g. *OsRacB*, *EDS5* and *OsBI-1* on chromosome 2) are concentrated in small chromosomal regions (Fig. 1). This may have important implications for marker-assisted selection.

Finally, some regions smaller than 500 kb were identified that contain either mapped *Pi* genes or blast metaQTLs. With the availability of insertions mutants (An et al. 2005), candidate gene approaches can now be initiated to identify genes responsible for *Pi* loci and blast metaQTLs.

The overview of the rice defense systems presented here indicates that some chromosomal regions are enriched for some classes of genes. For example, chromosome 12 is rich in *Pi* genes, chromosome 3 is rich in defense-related genes and the top of chromosome 2 in IDR genes. This is an important observation when considering large regions of the genome for marker-assisted selection.

We have highlighted rice regions that are most relevant for marker-assisted selection. These regions are represented by metaQTLs that are either robust (that is detected in most studies), or that are effective against a lot of the tested isolates of *M. oryzae* (Table 2). The markers provided for these metaQTLs (Table 2) are the first examples of the interest of combining genomic molecular data with genetic information for blast disease resistance.

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