Chapter 21
Rice Pathogens in Japan

Hideo Ishii

Abstract *Magnaporthe oryzae* causes blast, the most serious disease on rice. The fungus is genetically diverse and hence easily develops resistance to fungicides. In Japan, it started from the resistance to kasugamycin and has continued up until MBI-D and QoI fungicide resistance experienced most recently. The history of fungicide resistance occurred on rice blast disease and research and countermeasure taken in the country are summarized in this chapter. Some related information from overseas is also introduced.

Keywords Benzimidazole fungicides • Fungicide resistance • *Gibberella fujikuroi* • *Magnaporthe oryzae* • MBI-D fungicides • QoI fungicides • Rice Bakanae • Rice blast

21.1 Introduction

Rice is one of the most important crops worldwide. Blast, caused by the fungus *Magnaporthe oryzae*, is the most serious disease on rice. This disease is distributed in about 85 countries (Kato 2001). In Japan, applications of chemical fungicides are common to control rice diseases although alternative methods such as biofungicides, hot water seed treatment, and blast-resistant multiline rice cultivars are employed. Chemical control of rice diseases, blast disease in particular, has been reviewed recently (Hirooka and Ishii 2013). Fungicide resistance in rice was also reviewed before (Ishii 2011; Uesugi 1982).

Four decades have passed since fungicide resistance first occurred on rice in Japan that was the resistance of blast fungus *M. oryzae* to kasugamycin. Thereafter, resistance issue was not always very serious on rice although some other cases were reported as reviewed by Uesugi (1982). However, the new problem of resistance to

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MBI-D fungicides caused the decrease of control efficacy, and there was a concern whether QoI resistance might occur in the field populations of rice blast fungus in the future. In this paper, the history of fungicide resistance in rice and current topics related are reviewed.

21.2 Rice Blast Disease

21.2.1 Kasugamycin (Antibiotic)

In the early 1970s, kasugamycin was the most common fungicide for the control of this disease and sprayed frequently (Miura et al. 1975). However, control efficacy against this disease was lost in northern part of Japan in 1971 (Miura 1984).

Resistant isolates of *M. oryzae* exhibited reduced sensitivity to kasugamycin on rice-straw decoction agar medium. Control efficacy of kasugamycin was extremely low in the tests when resistant isolates were inoculated. However, resistant strains declined after withdrawal of this fungicide and control efficacy against blast was recovered gradually (Fukaya and Kobayashi 1982; Miura 1984).

21.2.2 Organophosphorus Fungicides

The fungicide IBP has been used since 1965 and resistance of *M. oryzae* to this fungicide was found in 1976 when the decrease of efficacy was observed (Katagiri et al. 1980; Yaoita et al. 1978). Two levels of IBP resistance were reported but most of the resistant isolates showed a moderate level of resistance. IBP-resistant isolates exhibited cross resistance to another organophosphorus fungicide EDDP and an organosulfur fungicide isoprothiolane (Katagiri and Uesugi 1977). The frequency of IBP-resistant strains also decreased when the selection pressure with the fungicide was removed (Iijima and Terasawa 1987).

21.2.3 MBI-D Fungicides

M. oryzae requires melanized appressoria for host penetration. MBI-D fungicides containing carpropamid, diclocymet, and fenoxanil (Fig. 21.1) inhibit scytalone dehydratase in fungal melanin biosynthesis. Nursery box treatment with MBI-D fungicides, carpropamid in particular, became common in many rice-growing areas as this fungicide exhibited long-lasting control efficacy against blast disease. The treatment was labor cost effective and greatly contributed to diminishing fungicide applications in paddy fields and lowering the pesticide input to the environment.

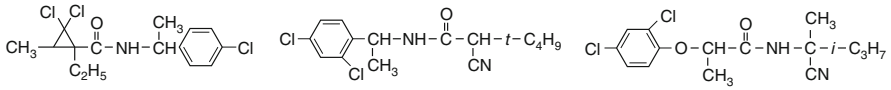

Fig. 21.1 MBI-D fungicides which inhibit scytalone dehydratase in fungal melanin biosynthesis

In 2001, however, the efficacy of carpropamid against leaf blast decreased suddenly in some areas of the southern part of Japan. Results from studies indicated that resistant strains appeared and played a significant role in the decrease of fungicide efficacy (Yamaguchi et al. 2002; Sawada et al. 2004). As of 2013, resistant strains have been detected in 36 out of 47 prefectures within the country although the impact of resistance greatly differed depending on the areas.

Results from monitoring tests suggested that resistant strains seemed to be less fit to the environment as their populations decreased in the absence of the selection pressure by MBI-D fungicides (Suzuki et al 2010; Yasunaga 2007). In a model experiment, resistant isolates showed lower competitive ability than sensitive isolates as the proportion of resistant ones decreased when their mixtures with sensitive isolates were inoculated repeatedly under no fungicide treatment (Kimura 2006).

Despite that, production of carpropamid was stopped and the use of diclocymet and fenoxanil in the same cross-resistance group has been largely reduced (Fig. 21.2). This is a typical example showing how seriously resistance development in major pathogens influences the market share of particular chemical control agents. In this case, MBI-D fungicides were replaced by disease resistance inducers such as probenazole, the inducer of systemic acquired resistance (SAR) commercially introduced first in the world, and two other products tiadinil and isotianil (Fig. 21.3). Probenazole has been widely used for 40 years as a major blasticide with no sign of field resistance development in *M. oryzae*. More details of MBI-D fungicide resistance of rice blast fungus are described in Chap. 11 of this book.

MBI-R fungicides, another class of melanin biosynthesis inhibitors, contain tricyclazole, pyroquilon, and phthalide and the primary target of these fungicides is 1,3,8-trihydroxynaphthalene reductase (Motoyama and Yamaguchi 2003). Laboratory mutants resistant to tricyclazole were obtained in rice blast fungus (Zhang et al. 2006); however, those mutants have not been isolated from the field where decreased efficacy of tricyclazole was reported in China (Zhang et al. 2009). No field isolates resistant to MBI-R fungicides have been found in Japan so far although nearly 30 years have passed since three fungicides in this class were registered and used for rice blast control (Eizuka et al. 2001). In Italy, tricyclazole-based fungicide was registered at the end of the 1990s and has been widely used for rice blast management since then. However, reduced sensitivity to tricyclazole has not been observed in the populations of *M. oryzae* collected from rice fields repeatedly treated with this fungicide over a 12-year period (Kunova et al. 2014).

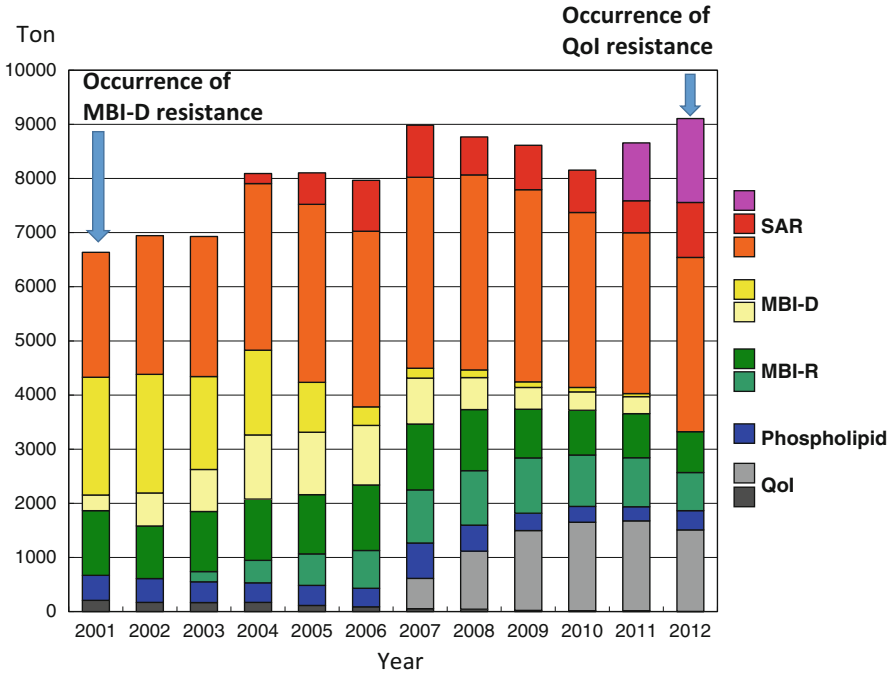

Fig. 21.2 Market value of fungicides used for rice blast in Japan

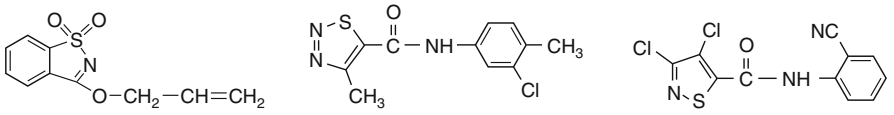

Fig. 21.3 Resistance inducers against rice diseases

21.2.4 QoI Fungicides

21.2.4.1 Background of Resistance Development

Occurrence and subsequent widespread of MBI-D resistance in rice blast fungus resulted in the reduction of the use of this class of fungicides rapidly. Alternatively, QoI fungicides became popular particularly when orysastrobin came into the market for nursery box treatment (Hirooka and Ishii 2013). Two other fungicides azoxystrobin and metominostrobin (Fig. 21.4) in the same cross resistance group had already been marketed for applications to paddy field. Nursery box treatment of rice with granule formulations of orysastrobin exhibited long-lasting control efficacy not only against blast but also against sheath blight diseases,

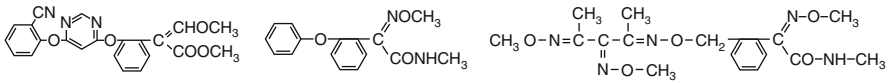

Fig. 21.4 QoI fungicides registered for rice disease control

caused by *Rhizoctonia solani*, contributing to the reduction of fungicide applications (Stammler et al. 2007).

It was well known that QoI fungicides possessed high risk for resistance development in target pathogens. In fact, resistance had occurred in many diseases on various crops other than rice within Japan (Ishii 2012). Laboratory mutants of blast fungus resistant to azoxystrobin were obtained on culture media amended with azoxystrobin and SHAM (salicylhydroxamic acid), an inhibitor of AOX (alternative oxidase) in electron transfer pathway of fungal mitochondria (Avila-Adame and Köller 2003). In those spontaneous mutants, point mutations such as G143A (substitution of glycine with alanine at position 143) were found in cytochrome *b* gene which encodes the fungicide target protein.

However, as experienced with MBI-R fungicides mentioned above, it was not certain yet whether QoI fungicide-resistant strains could appear in the field even if resistant mutants were produced in the laboratory. Subsequently, azoxystrobin-resistant strains were reported in *Pyricularia grisea* (*M. oryzae*), closely related with rice blast fungus, from perennial ryegrass grown in the USA where severe outbreaks of gray leaf spot were observed despite the treatment with this fungicide (Vincelli and Dixon 2002). Molecular characterization of resistance was conducted and two mutations, G143A and F129L (substitution of phenylalanine with leucine at position 129) of cytochrome *b* gene, were found in resistant isolates. The former mutation was involved in higher level of resistance to azoxystrobin and trifloxystrobin than the latter one (Kim et al. 2003).

21.2.4.2 Monitoring for Field Resistance on Rice

Monitoring for QoI fungicide sensitivity of rice blast fungus was started in Japan. Araki et al. (2005) first established baseline sensitivity to metominostrobin and then compared it with field isolates collected from 2001 to 2003 when no QoI-resistant isolates were found and QoI sensitivity occurred throughout Japan. In this study, neither G143A nor F129L mutations were observed in cytochrome *b* gene from isolates examined by PCR-RFLP analysis. In 2004 and 2005, Stammler et al. (2007) also monitored for resistance quantitatively using pyrosequencing method but both mutations, G143A and F129L, were not detected. Both of these groups concluded that no QoI-resistant isolates and full QoI-sensitive situation were found throughout Japan. Meanwhile, it was briefly reported that benomyl and azoxystrobin activity against rice blast decreased in field experiments conducted in Louisiana, the USA, over time suggesting the occurrence of resistance (Groth and Rush 2006).

Monitoring studies were also carried out in our laboratory using isolates collected from various regions in 2004 and 2005 but resistant isolates were never found in mycelial growth tests (Wei et al. 2009). Growth of all isolates was inhibited on PDA medium supplemented with 1 mg L^{-1} (a.i.) azoxystrobin plus 1 mM *n*-propyl gallate, an inhibitor of AOX known to be more specific than SHAM.

21.2.4.3 Guideline for Fungicide Use

When orysastrobin was marketed on rice, the Japan Fungicide Resistance Action Committee (J FRAC, http://www.jfrac.com/) made a guideline indicating how to use orysastrobin and other QoI fungicides which had already been in the market. Shortly after that, the Research Committee on Fungicide Resistance (http://www.taiseikin.jp/), the Phytopathological Society of Japan, also proposed a guideline on this subject (So and Yamaguchi 2008). It was proposed to use QoIs only once per year on rice if necessary. In the latter guideline, furthermore, QoIs were recommended to be used in alternation with other unrelated fungicides such as MBI-R fungicides or resistance inducers every 2–3 years when QoIs were employed in nursery box treatment. As rice blast fungus is disseminated not only by wind but also by seeds, it was not recommendable to use QoIs in a paddy field where commercial seeds were produced. The same strategies were also proposed for MBI-D fungicides, if they were still effective.

21.2.4.4 Occurrence of Resistance

Nakamura et al. (2008) showed that three isolates of blast fungus sampled in 2007 were less sensitive to azoxystrobin in inoculation to young rice plants. In the summer 2012 subsequently, heavy outbreak of leaf blast disease has been reported from various regions in the western part of Japan after nursery box was regularly treated with orysastrobin for some years. Results from experiments conducted urgently confirmed that QoI-resistant strains were distributed in a large populations of the fungus (Ishii and Fuji 2013; Miyagawa et al. 2013). Resistant strains have been reported officially from three prefectures, Yamaguchi, Shimane, and Ehime, in 2012. As of October 2014, the presence of resistant strains has been proved in 16 prefectures. Cross resistance among three QoI fungicides, orysastrobin, azoxystrobin, and metominostrobin, was confirmed by fungus inoculation tests (Miyagawa and Fuji 2013). Resistant isolates were clearly distinguished from sensitive ones on PDA medium supplemented with 1 mg L^{-1} (a. i.) azoxystrobin plus 1 mM *n*-propyl gallate (Fig. 21.5).

Monitoring for QoI fungicide sensitivity has been continued, but resistant strains have not been detected from some prefectures such as Nagasaki, and Hiroshima yet. In these areas, the authorities related give rice growers a caution on the use of QoI fungicides. Some prefectures have started adopting the guideline from the Research Committee on Fungicide Resistance described above.

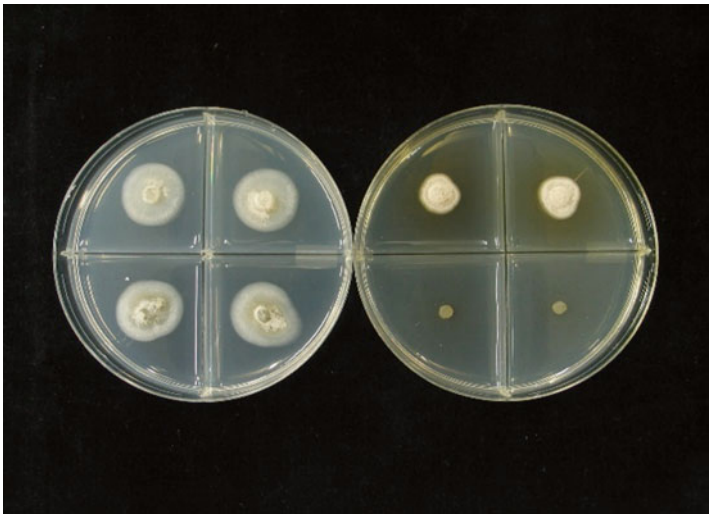

Fig. 21.5 Mycelial growth test for the detection of QoI-resistant isolates of rice blast fungus. *Upper*, resistant isolates; *lower*, sensitive isolates. *Left*, No fungicide treatment; *right*, 1 mg L^{-1} (a.i.) azoxystrobin plus 1 mM *n*-propyl gallate

21.2.4.5 Mechanism of Resistance

It is well known that QoI resistance is mostly caused by a single point mutation of fungicide-targeted cytochrome *b* gene in pathogens (Ishii 2012). In highly resistant isolates of the fungus *Pyricularia grisea* grown in the USA where there were severe outbreaks of gray leaf spot on perennial ryegrass, nucleotide sequences at the position 143 of this gene were converted from GGT to GCT resulting in the amino acid substitution of glycine (G) by alanine (A) (Kim et al. 2003). Another mutation, the change of phenylalanine (F) to leucine (L), was found at the position 129 in moderately resistant isolates.

When the sequence of cytochrome *b* gene was examined, QoI-resistant isolates of rice blast fungus carried the same mutation of G143A (exchange of GGT to GCT at position 143) as expected, but F129L mutation, found in the gray leaf spot fungus in the USA previously, was not recognized (Miyagawa et al. 2013). The partial nucleotide sequences analyzed in resistant and sensitive isolates are shown in Fig. 21.6 (Ishii 2014).

21.2.4.6 Molecular Diagnosis of Resistance

Using the single point mutation specifically found in cytochrome *b* gene of QoI-resistant isolates, a couple of methods have been developed to diagnose resistance. PCR-RFLP analysis is most widely used at present (Miyagawa and Fuji 2013). Fragments of cytochrome *b* gene are PCR-amplified from cultures of isolates with

Sensitive isolate:

5'…GGTTTCCTA……TTATGAGGTGCTACA……3'

Resistant isolate:

5'…GGTTTCCTA……TTATGAGCTGCTACA……3'

Fig. 21.6 A single point mutation at the cytochrome *b* gene found in QoI-resistant isolates of *Magnaporthe oryzae*. Nucleotide sequences corresponding to position 143 of cytochrome *b* gene are underlined. GGT at sensitive isolates are converted to GCT in resistant isolates resulting in the substitution of glycine by alanine

two primers designed by BASF and the products treated with a restriction enzyme *Fnu*4HI are loaded on an agarose gel. After digestion with this enzyme, the products from resistant isolates show two bands on a gel, whereas those from sensitive isolates remain a single band as they do not possess the restriction site (GCNGC) of *Fnu*4HI at the position 143 of cytochrome *b* gene.

The method of PCR-RFLP can also be applied for genomic DNA extracted from diseased rice leaves using microwave and pathogen-contaminated grains (Wei et al. 2009). Other methods such as ASPCR (allele-specific PCR) with a primer which recognizes the G143A mutation specifically and PCR-Luminex system suitable for high-throughput diagnosis were further developed (Wei et al. 2009). PCR-Luminex was originally introduced for rapidly identifying MBI-D fungicide resistance of rice blast fungus or identifying fungal species causing head blight of wheat (Ishii et al. 2008). In the future, the development of a more simple method like LAMP (loop-mediated isothermal amplification) may be necessary for molecular resistance monitoring on site.

Heteroplasmic status has often been found in cytochrome *b* gene of QoI-resistant fungal isolates (Ishii 2011). It is not known yet whether resistant isolates of rice blast fungus found in Japan carry heteroplasmic cytochrome *b* gene or not. If so, however, molecular methods for identifying QoI resistance would encounter some difficulties as experienced previously (Ishii 2010). Involvement of heteroplasmic cytochrome *b* gene with stability of resistance is described in details in Chap. 3 of this book.

21.2.4.7 Countermeasure with Resistance

In the areas where QoI-resistant strains were found to be distributed widely, the use of QoI fungicides has been stopped. However, in some cases, resistant strains have also been detected in a low frequency from the areas in which QoI fungicides were used in paddy fields only with foliar applications but not as a nursery box treatment and even from the areas with no use history of QoI fungicides. Therefore, it will be an important subject in the future to assess the resistance risk when these fungicides are used for foliar applications.

It is definitely important to save QoI fungicides within one application per year as well as to avoid their yearly successive applications for a nursery box treatment. There have been no reports on resistance to MBI-R fungicides and resistance inducers so far. Alternative use of QoI fungicides with these fungicides will be highly recommended to delay QoI resistance development.

As rice blast disease is disseminated not only by air but also by seeds, it is also quite important how we control seed contamination effectively. In general, it is recommended to growers not to use seeds harvested and stored by themselves and to alternatively purchase seeds disinfected by other sectors previously. However, it is not very rare to see those commercial seeds contain fungal strains resistant to fungicides. Such cases have been claimed on MBI-D as well as QoI fungicide resistance.

Due to this, the guideline recommends that QoI fungicides shouldn't be used in a seed-producing paddy field and its surroundings. In some regions actually, QoI fungicides are never applied through the course of rice seed production. Management such as removal of diseased plant debris, rice straws, and hulls from related facilities is also effective to sanitize the environment resulting in the decrease of infection source.

21.3 Report of QoI and SDHI Fungicide Resistance in Other Diseases Overseas

Occurrence of sheath blight disease, caused by *Rhizoctonia solani*, is increasing recently. Although it hasn't been found in Japan yet, resistance of this pathogen to azoxystrobin has been reported in the USA (Olaya et al. 2012). In addition, control failure using azoxystrobin has been mentioned in *Bipolaris* leaf spot disease on turf grass and reduced fungicide sensitivity of *B. spicifera* isolates was briefly reported (Tomaso-Peterson 2012). Although brown spot disease rarely occurs on rice these days in Japan, this disease is caused by *Cochliobolus miyabeanus* close to *B. spicifera*.

Most recently, QoI resistance has been reported in wheat blast pathogen *M. oryzae* in Brazil (Castroagudin et al. 2015). Control of this disease relied mainly on QoIs and these fungicides were used over the last 15 years. As a result, isolates carrying high frequency of the G143A mutation in cytochrome *b* gene associated with high QoI resistance have been sampled from both wheat and other poaceous host species of *M. oryzae* adjacent to wheat fields. Castroagudin et al. (2015) mentioned that these species may be an important reservoir for the pathogen that could contribute QoI-resistance inoculum during the early stage of a wheat blast epidemic. Furthermore, in *M. grisea*, anastomosis (i.e., hyphal fusion) has been proposed as a possible mechanism of resistance through transmission of the G143A mutation (Avila-Adame 2014).

New generation of succinate dehydrogenase-inhibiting (SDHI) fungicides has been recently developed very actively worldwide. Penflufen, one of them, has been registered in early 2014 for the control of sheath blight disease on rice in Japan.

SDHI fungicides also carry moderate to high risk for resistance development. In fact, isolates of *R. solani* resistant to the preexisting SDHI fungicide thifluzamide have been obtained in the laboratory (Mu et al. 2014). Furthermore, resistant isolates have been detected from the field as well and they reduced the efficacy of thifluzamide in fungus inoculation tests (Liu unpublished).

21.4 Fungicide Resistance in Bakanae Disease

21.4.1 Benzimidazole Fungicide Resistance

The benzimidazole fungicide benomyl, used in a mixture with thiram as a seed disinfectant, effectively controlled Bakanae disease caused by *Gibberella fujikuroi* (*Fusarium moniliforme*). However, isolates of this pathogen resistant to benomyl were detected in 1980 (Ogawa and Suwa 1981), and heavy occurrence of this disease due to resistance was reported in 1984. In 1987, resistant strains were widely distributed in 37 prefectures in Japan (Yoshino 1988).

Recently, Suga et al. (2013) divided the isolates of *F. fujikuroi* complex into two groups: G strains, gibberellic acid producing, thiophanate-methyl resistant, and highly ipconazole sensitive, and F strains, not gibberellic acid producing, thiophanate-methyl sensitive, and less ipconazole sensitive. Molecular mechanism has been studied and resistance was found to result from mutations in β_2*tub* gene [GAG (Glu) → GTG (Val) at codon 198 and TTC (Phe) → TAC (Tyr) at codon 200] but not in β_1*tub* gene (Chen et al. 2014). This finding could explain the following reports from early studies on fungicide sensitivity and binding to target proteins: (1) increased sensitivity (negative cross-resistance) to the *N*-phenylformamidoxime compound *N*-(3,5-dichloro-4-propynyloxyphenyl)-N′-methoxyformamidine (DCPF) was associated with a high level of carbendazim resistance in *Botrytis cinerea* but not with a moderate resistance level and (2) increased sensitivity to DCPF was not observed in carbendazim-resistant isolates of *G. fujikuroi* (Ishii and Takeda 1989).

21.4.2 DMI Fungicide Resistance

21.4.2.1 Reduced Sensitivity in Japan

G. fujikuroi isolates less sensitive to the DMI fungicide triflumizole were detected. MIC values of this fungicide for mycelial growth on PDA were 1,000 mg L^{-1} or more, but the EC_{50} values were less than 1.3 mg L^{-1}, only slightly different from sensitive isolates (Hamamura et al. 1989). Less triflumizole-sensitive isolates reduced pathogenicity remarkably against rice seeds and flowers than sensitive isolates, and such a difference in pathogenicity coincided with their lower production

of gibberellic acids. Sensitivity of this fungus was also tested for pefurazoate, and less triflumizole-sensitive isolates were also less sensitive to this DMI fungicide, but pefurazoate still showed high efficacy against these isolates in artificial inoculation tests (Wada et al. 1990).

Similarly, isolates with MIC values lower than 0.78 mg L^{-1} for ipconazole were pathogenic but all the isolates with MIC values higher than or equal to 1.56 mg L^{-1} were not pathogenic to rice seedlings. Low pathogenicity or lack of pathogenicity of the isolates less sensitive to ipconazole may contribute to the stable efficacy of this fungicide (Tateishi and Chida 2000). Subsequently, *G. fujikuroi* species complex was classified into two groups based on MIC of ipconazole: (1) between 0.10 and 0.78 mg L^{-1} and gibberellic acid-producing *F. fujikuroi* and (2) 0.78 to 6.25 mg L^{-1} and no gibberellic acid-producing species such as *F. proliferatum* (Tateishi et al. 2011). Most recently, *G. fujikuroi* isolates resistant to benomyl and less sensitive to DMIs were predominant, and these isolates were pathogenic to rice causing a disease on fungicide-treated seeds (Kudo et al. 2014).

21.4.2.2 Resistance Development in Korea

In Korea, the incidence of Bakanae disease has increased rapidly. Resistance of the pathogen to prochloraz and hexaconazole was detected using the agar dilution method, but there was no evidence of cross-resistance between these two DMI fungicides (Jeong et al. 2009). Subsequently, cross-resistance to prochloraz and tebuconazole was found in some isolates (Lee et al. 2010). Surprisingly, it was reported that degradation of prochloraz might account for the reduced sensitivity to this fungicide (Kim et al. 2010). Relationship of *in vitro* resistance with disease control in the field has not been reported yet in details.

21.5 Recent Topics on Rice Disease Control

There is a strong demand from consumers to reduce pesticide applications. The use of biofungicides and/or hot water treatment has been introduced for seed disinfection of rice, but these treatments tend to increase the occurrence of Bakanae disease. Therefore, the development of alternative methods is still required to control major diseases.

'Koshihikari', the most popular and abundantly grown rice cultivar in Japan, was crossed with a resistant cultivar and progenies were further crossed with 'Koshihikari' five to six times. In 2005, seeds of four blast-resistant multi-lines thus bred were mixed together and cultivated at ca. 80 % of paddy fields in Niigata Prefecture, the most important rice-growing region in Japan. As a result, the occurrence of leaf and panicle blast dramatically decreased and blasticide applications were reduced to one fourth as compared before (Ishizaki 2010).

A mixture of the QoI fungicide orysastrobin and the best-selling disease resistance inducer probenazole (plus an insecticide) was developed and commercialized locally. These strategies are expected to play roles in reducing the risk of fungicide resistance.

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