Evolution of an Avirulence Gene, *AVR1-CO39*, Concomitant with the Evolution and Differentiation of *Magnaporthe oryzae*

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The significance of *AVR1-CO39*, an avirulence gene of the blast fungus corresponding to *Pi-CO39(t)* in rice cultivars, during the evolution and differentiation of the blast fungus was evaluated by studying its function and distribution in *Pyricularia* spp. When the presence or absence of *AVR1-CO39* was plotted on a dendrogram constructed from ribosomal DNA sequences, a perfect parallelism was observed between its distribution and the phylogeny of *Pyricularia* isolates. *AVR1-CO39* homologs were exclusively present in one species, *Pyricularia oryzae*, suggesting that *AVR1-CO39* appeared during the early stage of evolution of *P. oryzae*. Transformation assays showed that all the cloned homologs tested are functional as an avirulence gene, indicating that selection has maintained their function. Nevertheless, *Oryza* isolates (isolates virulent on *Oryza* spp.) in *P. oryzae* were exceptionally noncarriers of *AVR1-CO39*. All *Oryza* isolates suffered from one of the two types of known rearrangements at the *Avr1-CO39* locus (i.e., G type and J type). These types were congruous to the two major lineages of *Oryza* isolates from Japan determined by MGR586 and MAGGY. These results indicate that *AVR1-CO39* was lost during the early stage of evolution of the *Oryza*-specific subgroup of *P. oryzae*. Interestingly, its corresponding resistance gene, *Pi-CO39(t)*, is not widely distributed in *Oryza* spp.

The genus *Pyricularia* is the causal agent of blast disease of various monocot species. This fungal genus includes several species, such as *Pyricularia zingiberi*, pathogenic on *Zingiber* spp., and *P. higginsii*, pathogenic on *Cyperus* spp. The most familiar among them is *P. grisea* (Rossman et al. 1990) (teleomorph, *Magnaporthe grisea* (Hebert) Barr.), which has caused destructive epidemics on staple gramineous crops. *P. grisea* is isolated from rice (*Oryza sativa*), foxtail millet (*Setaria italica*), common millet (*Panicum miliaceum*), finger millet (*Eleusine coracana*), wheat (*Triticum aestivum*), perennial ryegrass (*Lolium perenne*), crabgrass (*Digitaria sanguinalis*), and so on. However, isolates from each plant genus constitute a host-specific subgroup. For example, isolates from rice are exclusively pathogenic on *Oryza* spp., whereas those from foxtail millet are exclusively pathogenic on *Setaria* spp. (Kato et al. 2000; Tosa et al. 2004). In this article, we describe these isolates as *Oryza* isolates, *Setaria* isolates, and so on. Kato and

associates (2000) found that *Oryza*, *Setaria*, *Panicum*, *Triticum*, and *Eleusine* isolates form a genetically close, interfertile group (CC group), and are distinct from *Digitaria* isolates, to which the Latin name *P. grisea* was first assigned. Based on this finding, Kato and associates (2000) suggested that the CC group is a species distinct from *P. grisea* and should be referred to as *P. oryzae*. Based on a multilocus phylogenetic study, Couch and Kohn (2002) recognized and formally described a new species, *M. oryzae*. The species boundary of *M. oryzae* was perfectly congruous to that of *P. oryzae* proposed by Kato and associates (2000).

AVR1-CO39 is a gene of the blast fungus that specifies avirulence to rice cv. CO39 (Smith and Leong 1994; Valent et al. 1991). Its function depends on a resistance gene in CO39 (*Pi-CO39(t)*) (Chauhan et al. 2002), and is assumed to follow the gene-for-gene interaction (Flor 1956). Farman and Leong (1998) cloned a 1.05-kb fragment containing *AVR1-CO39* by chromosome walking from linked restriction fragment length polymorphism markers. Subsequently, Farman and associates (2002) found that all rice-infecting isolates (*Oryza* isolates) tested had rearrangements at the *Avr1-CO39* locus that rendered this locus nonfunctional. This finding provided a molecular explanation for why most *Oryza* isolates are virulent on CO39. However, the significance of *AVR1-CO39* in the natural *Pyricularia* population is still unclear because 4091-5-8 and 2539, the *Pyricularia* strains in which *AVR1-CO39* was identified (Smith and Leong 1994; Valent et al. 1991), were laboratory strains developed in breeding programs.

In the present study, we examined the distribution and function of *AVR1-CO39* in *Pyricularia* populations together with the distribution and function of *Pi-CO39(t)* in *Oryza* populations. The results provide new insight into the significance of the *AVR1-CO39*–*Pi-CO39(t)* interaction during the evolution of *P. oryzae* (*M. oryzae*).

RESULTS

Distribution of *AVR1-CO39* in *Pyricularia* populations.

Farman and Leong (1998) attributed the *AVR1-CO39* avirulence function to a 1.05-kb DNA fragment containing several potential open reading frames. Here, we define *AVR1-CO39* as the minimal 1.05-kb fragment that conferred avirulence in transformation assays. Structures of the *Avr1-CO39* locus in the laboratory strain 2539 (W type) and *Oryza* isolates (G type and J type) that were revealed by Farman and associates (2002) are shown in Figure 1. Actually, they found three types of rearrange-

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ments among *Oryza* isolates (i.e., G1 type, G2 type, and J type). Here, the G1 and G2 types are designated together as G type because they share the same rearrangement; that is, an approximately 20-kb deletion including the entire *AVR1-CO39*. By contrast, the J type is characterized by a deletion of the 5' half of *AVR1-CO39* as well as point mutations (Farman et al. 2002).

To survey the distribution of *AVR1-CO39* in *Pyricularia* populations, a total of 82 isolates were chosen from stock cultures in the Laboratory of Plant Pathology, Kobe University, so that they covered the entire diversity of the stock cultures (Table 1). In a dendrogram constructed from nucleotide sequences of the ribosomal DNA (rDNA) internal transcribed spacer (ITS)1-5.8S-ITS2 region, isolates belonging to *P. oryzae* (*M. oryzae*) formed a strongly supported branch with a very high bootstrap value, 100 (Fig. 2), supporting the idea that these isolates constitute a single species (Couch and Kohn 2002; Kato et al. 2000). Within *P. oryzae*, *Oryza* isolates clustered into a single subgroup with a high bootstrap value, 89 (Fig. 2).

When genomic DNA of the 82 isolates was hybridized with the 3' half of *AVR1-CO39*, strong signals were detected in *P. oryzae* isolates but not in the other *Pyricularia* spp. (Figs. 2 and 3A). In *P. oryzae*, *Oryza* isolates almost exclusively were noncarriers (Fig. 2). Some *Oryza* isolates showed a strong signal (Figs. 2 and 3A), but polymerase chain reaction (PCR) analysis suggested that these isolates do not carry the entire

AVR1-CO39 (Figs. 2 and 3B). A further PCR analysis was used to determine the type of rearrangements (Farman et al. 2002) and revealed that the *Oryza* isolates with a strong signal (Ken54-20, Hoku 1, Ken53-33, and 2012-1) have the J-type structure (data not shown). It should be noted that an isolate from Vietnam (VHT6.1) had the J-type structure with some modification (insertion of a 0.5-kb fragment around the breakpoint). All the other *Oryza* isolates with no signals had the G-type structure (data not shown).

In summary, *AVR1-CO39* homologs were confined within one species, *P. oryzae*, but *AVR1-CO39* from all *Oryza* isolates of *P. oryzae* suffered from the rearrangements leading to the loss of function. These results suggest that *AVR1-CO39* was present in the early stages of evolution of *P. oryzae* and that, in turn, the rearrangements at the *AVR1-CO39* locus occurred in the early stages of evolution of the *Oryza* subgroup in *P. oryzae*.

Relationship between lineages and the structures of the *Avr1-CO39* locus in *Oryza* isolates.

If the rearrangements at the *Avr1-CO39* locus actually occurred in the early stage of evolution of the *Oryza* subgroup, there should be a close relationship between lineages in the *Oryza* subgroup and the types of rearrangements at the *Avr1-CO39* locus. Don and associates (1999a) examined the population structure of *Oryza* isolates collected in Japan using

Table 1. *Pyricularia* isolates tested

Species	Isolates	Host (abbreviation)	Locality	Collector
<i>Pyricularia oryzae</i>	Guy11	<i>Oryza sativa</i> (Osa)	Guiana	J. L. Notteghem
<i>P. oryzae</i>	Ken 54-04	<i>O. sativa</i> (Osa)	Japan (Gifu)	S. Kiyosawa et al.
<i>P. oryzae</i>	Ken 54-20	<i>O. sativa</i> (Osa)	Japan (Yamaguchi)	S. Kiyosawa et al.
<i>P. oryzae</i>	Hoku 1	<i>O. sativa</i> (Osa)	Japan (Hokkaido)	S. Kiyosawa et al.
<i>P. oryzae</i>	Ina 72	<i>O. sativa</i> (Osa)	Japan (Nagano)	S. Kiyosawa et al.
<i>P. oryzae</i>	Ina 168	<i>O. sativa</i> (Osa)	Japan (Aichi)	S. Kiyosawa et al.
<i>P. oryzae</i>	Ken 53-33	<i>O. sativa</i> (Osa)	Japan (Aichi)	S. Kiyosawa et al.
<i>P. oryzae</i>	P-2b	<i>O. sativa</i> (Osa)	Japan (Niigata)	S. Kiyosawa et al.
<i>P. oryzae</i>	2012-1	<i>O. sativa</i> (Osa)	Japan (Ishikawa)	M. Yamada et al.
<i>P. oryzae</i>	2403-1.	<i>O. sativa</i> (Osa)	Japan (Mie)	M. Yamada et al.
<i>P. oryzae</i>	1836-3	<i>O. sativa</i> (Osa)	Japan (Niigata)	M. Yamada et al.
<i>P. oryzae</i>	88A	<i>O. sativa</i> (Osa)	Japan (Ibaraki)	M. Yamada et al.
<i>P. oryzae</i>	CHNOS 59-6-1	<i>O. sativa</i> (Osa)	China (Yunnan)	H. Hayashi
<i>P. oryzae</i>	CHNOS 60-8-1	<i>O. sativa</i> (Osa)	China (Yunnan)	H. Hayashi
<i>P. oryzae</i>	Br10	<i>O. sativa</i> (Osa)	Brazil (Paulo Frontin)	S. Igarashi
<i>P. oryzae</i>	Br13	<i>O. sativa</i> (Osa)	Brazil (Campo Mourao)	S. Igarashi
<i>P. oryzae</i>	Br15	<i>O. sativa</i> (Osa)	Brazil (Tuppassi)	S. Igarashi
<i>P. oryzae</i>	Br18	<i>O. sativa</i> (Osa)	Brazil (Campo Mourao)	S. Igarashi
<i>P. oryzae</i>	PO-02-7501	<i>O. sativa</i> (Osa)	Indonesia (Tasikmalaya)	A. Mukelar
<i>P. oryzae</i>	PO-04-7501	<i>O. sativa</i> (Osa)	Indonesia (Tulungagung)	A. Mukelar
<i>P. oryzae</i>	PO-12-7301-2	<i>O. sativa</i> (Osa)	Indonesia (Tamanbogo)	A. Mukelar
<i>P. oryzae</i>	PO-12-7301	<i>O. sativa</i> (Osa)	Indonesia (Tamanbogo)	A. Mukelar
<i>P. oryzae</i>	VHT6.1	<i>O. sativa</i> (Osa)	Vietnam (Ha Tay)	L. Don
<i>P. oryzae</i>	VTB6.1	<i>O. sativa</i> (Osa)	Vietnam (Thai Binh)	L. Don
<i>P. oryzae</i>	VHG4.5	<i>O. sativa</i> (Osa)	Vietnam (Hau Giang)	L. Don
<i>P. oryzae</i>	VHT3.3	<i>O. sativa</i> (Osa)	Vietnam (Ha Tay)	L. Don
<i>P. oryzae</i>	GFSI1-7-2	<i>Setaria italica</i> (Sit)	Japan (Gifu)	H. Kato
<i>P. oryzae</i>	NRSI2-2-2	<i>S. italica</i> (Sit)	Japan (Nara)	H. Kato
<i>P. oryzae</i>	NRSI3-1-1	<i>S. italica</i> (Sit)	Japan (Nara)	H. Kato
<i>P. oryzae</i>	NNSI3-2-1	<i>S. italica</i> (Sit)	Japan (Nagano)	N. Hayashi
<i>P. oryzae</i>	IN77-16-1-1	<i>S. italica</i> (Sit)	India (Mysore)	H. Kato
<i>P. oryzae</i>	IN77-20-1-1	<i>S. italica</i> (Sit)	India (Mysore)	H. Kato
<i>P. oryzae</i>	KANSV1-4-1	<i>S. viridis</i> (Svi)	Japan (Kanagawa)	H. Kato
<i>P. oryzae</i>	NI913	<i>S. viridis</i> (Svi)	Japan (Chiba)	N. Nishihara
<i>P. oryzae</i>	NNPM1-2-1	<i>Panicum miliaceum</i> (Pmi)	Japan (Nagano)	N. Hayashi
<i>P. oryzae</i>	NRPM1-1-1	<i>P. miliaceum</i> (Pmi)	Japan (Nara)	H. Kato
<i>P. oryzae</i>	STPM1-3-2	<i>P. miliaceum</i> (Pmi)	Japan (Saitama)	H. Kato
<i>P. oryzae</i>	STPM4-2-2	<i>P. miliaceum</i> (Pmi)	Japan (Saitama)	H. Kato
<i>P. oryzae</i>	SZPM1-1-1	<i>P. miliaceum</i> (Pmi)	Japan (Shizuoka)	H. Kato
<i>P. oryzae</i>	YNPM4-1-1	<i>P. miliaceum</i> (Pmi)	Japan (Yamanashi)	H. Kato
<i>P. oryzae</i>	NI922	<i>P. bisulcatum</i> (Pbi)	Japan (Tochigi)	N. Nishihara
<i>P. oryzae</i>	G10-1	<i>Eleusine coracana</i> (Eco)	Japan (Tochigi)	S. Itoi et al.

(continued on next page)

MGR586 (Hamer et al. 1989) and MAGGY (Farman et al. 1996), and found that Japanese isolates collected after 1970 are grouped into two lineages, JL1 and JL2, at the similarity level of 0.7. The structure at the *Avr1-CO39* locus in these *Oryza* isolates is summarized in Table 2 with relevant data that

have been reported previously (Don et al. 1999a; Farman et al. 2002). A perfect congruence was recognized between the lineages and the types at the *Avr1-CO39* locus; all the JL1 isolates had the G-type structure whereas all the JL2 isolates had the J-type structure (Table 2).

Fig. 1. Allele structure of the *Avr1-CO39* locus in *Pyricularia oryzae* (Farman et al. 2002) and locations of primers used in this study. *AVR1-CO39* homologs in *Pyricularia* isolates were first screened by Southern hybridization using a 3' half fragment amplified with primers AVF2 and AVR1-2 as a probe. The second screening was performed by polymerase chain reaction (PCR) with primers AVF1-2 and AVR1-2. The other primers were used for PCR cloning of *AVR1-CO39* homologs.

Table 1. (continued from preceding page)

Species	Isolates	Host (abbreviation)	Locality	Collector
<i>P. oryzae</i>	Z2-1	<i>E. coracana</i> (Eco)	Japan (Kagawa)	S. Itoi et al.
<i>P. oryzae</i>	MZ5-1-6	<i>E. coracana</i> (Eco)	Japan (Miyazaki)	H. Kato
<i>P. oryzae</i>	Ken15-15-1	<i>E. coracana</i> (Eco)	Japan (Tokyo)	H. Kato
<i>P. oryzae</i>	SZEC1-1-1	<i>E. coracana</i> (Eco)	Japan (Shizuoka)	H. Kato
<i>P. oryzae</i>	GFEC1-5-1	<i>E. coracana</i> (Eco)	Japan (Gifu)	H. Kato
<i>P. oryzae</i>	IN77-31-1-1	<i>E. coracana</i> (Eco)	India (Bangalore)	H. Kato
<i>P. oryzae</i>	IN77-39-1-2	<i>E. coracana</i> (Eco)	India (Bangalore)	H. Kato
<i>P. oryzae</i>	NP10-17-4-1-3	<i>E. coracana</i> (Eco)	Nepal (Syabru)	S. Sakamoto
<i>P. oryzae</i>	NP10-28-1-1-1	<i>E. coracana</i> (Eco)	Nepal (Pokara)	S. Sakamoto
<i>P. oryzae</i>	NI1006	<i>E. africana</i> (Eaf)	Japan (Tochigi)	N. Nishihara
<i>P. oryzae</i>	NI1011	<i>E. boranensis</i> (Ebo)	Japan (Tochigi)	N. Nishihara
<i>P. oryzae</i>	IN77-36-1-1	<i>E. indica</i> (Ein)	India (Mysore)	H. Kato
<i>P. oryzae</i>	Br58	<i>Avena sativa</i> (Asa)	Brazil (Itambe)	A. S. Urashima
<i>P. oryzae</i>	Br3	<i>Triticum aestivum</i> (Tae)	Brazil (Londrina)	S. Igarashi
<i>P. oryzae</i>	Br7	<i>T. aestivum</i> (Tae)	Brazil (Jaguapita)	S. Igarashi
<i>P. oryzae</i>	Br8	<i>T. aestivum</i> (Tae)	Brazil (B. V. Paraiso)	S. Igarashi
<i>P. oryzae</i>	Br48	<i>T. aestivum</i> (Tae)	Brazil (Itapora)	S. Igarashi
<i>P. oryzae</i>	Br49	<i>T. aestivum</i> (Tae)	Brazil (Dourados)	S. Igarashi
<i>P. oryzae</i>	Br115.7	<i>T. aestivum</i> (Tae)	Brazil (Cornelio)	A. S. Urashima
<i>P. oryzae</i>	Br116.5	<i>T. aestivum</i> (Tae)	Brazil (Santa Mariana)	A. S. Urashima
<i>P. oryzae</i>	Br118.2D	<i>T. aestivum</i> (Tae)	Brazil (Panema)	A. S. Urashima
<i>P. oryzae</i>	TP1	<i>Lolium perenne</i> (Lpe)	Japan (Tochigi)	T. Tani
<i>P. oryzae</i>	TP2	<i>L. perenne</i> (Lpe)	Japan (Tochigi)	T. Tani
<i>P. oryzae</i>	AK-1	<i>L. perenne</i> (Lpe)	Japan (Akita)	T. Tani
<i>P. oryzae</i>	LW-3	<i>L. perenne</i> (Lpe)	Japan (Yamanashi)	T. Tani
<i>P. oryzae</i>	FI-5	<i>L. perenne</i> (Lpe)	Japan (Chiba)	T. Tani
<i>P. oryzae</i>	WK3-1	<i>L. perenne</i> (Lpe)	Japan (Yamaguchi)	T. Tani
<i>P. oryzae</i>	Br35	<i>Brachiaria plantaginea</i> (Bpl)	Brazil (Vilha Velha)	S. Igarashi
<i>P. oryzae</i>	Bp3a	<i>B. plantaginea</i> (Bpl)	Brazil (Sao Paulo)	A. S. Urashima
<i>P. oryzae</i>	NI986	<i>Eragrostis lehmanniana</i> (Ele)	Japan (Kumamoto)	N. Nishihara
<i>Pyricularia</i> sp.	NI919	<i>Leersia oryzoides</i> (Lor)	Japan (Chiba)	N. Nishihara
<i>Pyricularia</i> sp.	NI981	<i>Cenchrus ciliaris</i> (Cci)	Japan (Kumamoto)	N. Nishihara
<i>Pyricularia</i> sp.	Br36	<i>C. echinatus</i> (Cec)	Brazil (Alvorada do Sal)	S. Igarashi
<i>P. grisea</i>	Dig41	<i>Digitaria sanguinalis</i> (Dsa)	Japan (Hyogo)	Y. Iwamoto
<i>P. grisea</i>	NI907	<i>D. sanguinalis</i> (Dsa)	Japan (Tochigi)	N. Nishihara
<i>P. grisea</i>	IBDS4-1-1	<i>D. sanguinalis</i> (Dsa)	Japan (Ibaraki)	N. Hayashi
<i>P. grisea</i>	NI980	<i>D. smutsii</i> (Dsm)	Japan (Kumamoto)	N. Nishihara
<i>P. grisea</i>	Br29	<i>D. horizontalis</i> (Dho)	Brazil (Pedrinhas)	S. Igarashi
<i>Pyricularia</i> sp.	INA-B-92-45	<i>Sasa</i> sp. (Ssp)	Japan (Aichi)	S. Koizumi
<i>P. zingiberi</i>	HYZIM101-1-1-1	<i>Zingiber mioga</i> (Zmi)	Japan (Hyogo)	M. Ogawa
<i>Pyricularia</i> sp.	2539	Laboratory strain	...	...

Fig. 2. Distribution of *AVR1-CO39* on a dendrogram of *Pyricularia* isolates constructed from nucleotide sequences of the ribosomal DNA internal transcribed spacer (ITS)1-5.8S-ITS2 region. Original hosts of the isolates are shown in square brackets in abbreviations. Bootstrap values are indicated on the corresponding node for each cluster; + = present and - = absent.

Similar results were obtained with isolates collected before 1960; a JL1 isolate (Ken54-04) had the G-type structure whereas JL2 isolates (Ken54-20 and Ken53-33) had the J-type structure (Table 2). Some isolates were not grouped into JL1 or JL2 at the similarity level of 0.7, but most of such “orphan” isolates were clustered with either JL1 or JL2 at the similarity level of 0.6 (Don et al. 1999a). They tentatively were designated as cJL1 isolates and cJL2 isolates, respectively. Interestingly, cJL1 isolates (Ina72 and Ina168) also had the G-type structure whereas a cJL2 isolate (Hoku 1) had the J-type structure (Table 2). These results suggest that the allele types at the *AvrI-CO39* locus are congruous to the fundamental grouping of isolates determined at the lower similarity level (0.6). There was an exceptional isolate (P-2b) that was not clustered with either JL1 or JL2 even at the similarity level of 0.6 (Don et al. 1999a). It should be noted that no relationships was found between the allele type at the *AvrI-CO39* locus and the race code (i.e., the virulence spectrum on rice differentials) (Table 2).

Function of the *AVR1-CO39* homologs in *P. oryzae* isolates other than *Oryza* isolates.

To determine whether the *AVR1-CO39* homologs in *P. oryzae* isolates retain the avirulence function, 1.7- to 3.9-kb

fragments containing the homologs were amplified from genomic DNA of GFS11-7-2 (*Setaria* isolate), NNPM1-2-1 (*Panicum* isolate), MZ5-1-6 (*Eleusine* isolate), GFEC1-5-1 (*Eleusine* isolate), Br48 (*Triticum* isolate), Br58 (*Avena* isolate), and FI-5 (*Lolium* isolate) with primers shown in Table 3, and inserted into pBluescriptII SK(+). The nucleotide sequences of the 1.05-kb *AVR1-CO39* region in these clones were 98.0 to 99.8% identical to that of the functional fragment in 2539 (GenBank accession number AF463528).

These clones were introduced into PO-12-7301-2, an *Oryza* isolate with the G-type rearrangement, together with pSH75 containing a hygromycin resistance gene. The resulting transformants with hygromycin resistance were subjected to Southern hybridization analyses to confirm the integration of the transgenes. An example is shown in Figure 4. PAS1-3-1 through PAS1-3-15 are hygromycin-resistant cultures produced by the transformation of PO-12-7301-2 with pAS1-3 containing a 3.8-kb fragment derived from the *Setaria* isolate. Genomic DNAs of these cultures were double digested with *XhoI* and *NotI*, whose recognition sites are present in the multicloning site of pBluescriptII SK(+) and flanking the *EcoRV* site containing the insert, and were hybridized with the 3' half of *AVR1-CO39* derived from 2539. All the transformants had a 3.8-kb fragment hybridizing with *AVR1-CO39* (Fig. 4A), suggesting that they

Fig. 3. Distribution of *AVR1-CO39* in *Pyricularia* spp. **A**, Genomic DNA was digested with *Bam*HI and hybridized with the 3' half (500 bp) of the 1.05-kb *AVR1-CO39* fragment. **B**, Genomic DNA was amplified with the primer pair AVF1-2-AVR1-2.

carry at least one copy of the full-length transgene. These transformants also had additional bands with various sizes (Fig. 4A). To examine the copy number, genomic DNAs were double digested with *Xho*I and *Eco*RV. The *Xho*I site is present in the multicloning site in the plasmid flanking the cloned PCR fragment, whereas the *Eco*RV site in the multicloning site should have been lost due to the integration of the insert. Furthermore, the 3.8-kb insert does not have an *Eco*RV site. Therefore, the double digestion should liberate various sizes of fragments from the *Xho*I site on the integrated plasmids to *Eco*RV sites on the genomic DNA. The copy number of the *AVR1-CO39* fragment

varied among the transformants. For example, PAS1-3-1 had only two copies whereas PAS1-3-3 had multiple copies (Fig. 4B).

Two transformants were chosen arbitrarily from each population of hygromycin-resistant cultures with the same plasmid. A total of 14 transformants with the *AVR1-CO39* homologs derived from *P. oryzae* wild isolates were subjected to inoculation tests together with a transformant (PAX7-17-7) carrying the authentic *AVR1-CO39* derived from the laboratory strain 2539 and a control transformant (PBEv) carrying the pBluescriptII SK(+) empty vector. PO-12-7301-2 and the con-

Table 2. Relationship between lineages and the structure at the *Avr1-CO39* locus in *Oryza* isolates collected in Japan

Isolates	Locality	Year	Lineage ^a	Race ^b	Structure at the <i>AVR1-CO39</i> locus
Collected after 1970					
0124-3	Hokkaido	1976	JL1	037	G type ^c
0401-4	Miyagi	1976	JL1	107	G type ^c
0528-2	Akita	1976	JL1	000	G type
0616-4	Unknown	1976	JL1	307	G type
1801-4	Niigata	1976	JL1	007	G type
1836-3	Niigata	1976	JL1	033	G type ^c
2201-1	Gifu	1976	JL1	037	G type
2403-1	Mie	1976	JL1	007	G type ^c
3103-2	Tottori	1976	JL1	103	G type
4203-1	Nagasaki	1976	JL1	303	G type ^c
4403-2	Unknown	1976	JL1	101	G type
4411-2	Ooita	1976	JL1	113	G type ^c
4603-4	Kagoshima	1976	JL1	033	G type ^c
5A	Hyogo	1976	JL1	400	G type
1106-2	Saitama	1976	JL2	037	J type ^c
1117-4	Saitama	1976	JL2	007	J type ^c
1601-3	Nagano	1976	JL2	007	J type ^c
2303-1	Aichi	1976	JL2	033	J type ^c
Collected before 1960					
Ken 54-04	Gifu	1954	JL1	003	G type
Ina 72	Nagano	1957	cJL1	031	G type
Ina 168	Aichi	1958	cJL1	101	G type
Ken 54-20	Yamaguchi	1954	JL2	003	J type
Ken 53-33	Aichi	1953	JL2	137	J type
Hoku 1	Hokkaido	1948	cJL2	007	J type
P-2b	Niigata	1948	...	303	G type

^a Reported by Don and associates (1999a). cJL1 and cJL2 represent isolates that were clustered with JL1 and JL2 isolates, respectively, at the similarity level of 0.6.

^b Yamada and associates (1976).

^c Reported by Farman and associates (2002).

Table 3. Transformants of a rice isolate (PO-12-7301-2) with *AVR1-CO39* homologs and their virulence on rice cvs. CO39 and Yashimochi

Isolate or its transformant	Transgene					Infection type ^a	
	Plasmid	Origin of the insert ^b	Primers ^c	Insert size (kb)	Copy no.	CO39	Yashimochi
PO-12-7301-2	...	...	...	...	...	4	4
PBEv	pBluescriptII SK(+)	...	...	...	...	4	4
PAX7-17-7	pAX7-17	2539	WF1-WR1	3.9	5	1	4
PAS1-3-1	pAS1-3	GFSI1-7-2 (Sit)	WF9-WR1	3.8	2	1	4
PAS1-3-4	pAS1-3	GFSI1-7-2 (Sit)	WF9-WR1	3.8	3	1	4
PAP1-5-12	pAP1-5	NNPM1-2-1 (Pmi)	WF1-WR1	3.9	2	1	4
PAP1-5-15	pAP1-5	NNPM1-2-1 (Pmi)	WF1-WR1	3.9	3	1	3
PAE3-10-7	pAE3-10	MZ5-1-6 (Eco)	WF1-WR1	3.9	3	1	4
PAE3-10-13	pAE3-10	MZ5-1-6 (Eco)	WF1-WR1	3.9	3	1	4
PAE6-2-12	pAE6-2	GFEC1-5-1 (Eco)	WF1-WR1	3.9	3	1	4
PAE6-2-17	pAE6-2	GFEC1-5-1 (Eco)	WF1-WR1	3.9	3	2	4
PAT4-1-4	pAT4-1	Br48 (Tae)	WF6-WR4	1.7	2	2	4
PAT4-1-8	pAT4-1	Br48 (Tae)	WF6-WR4	1.7	4	1	4
PAA1-7-8	pAA1-7	Br58 (Asa)	WF9-WR1	3.8	2	0	4
PAA1-7-11	pAA1-7	Br58 (Asa)	WF9-WR1	3.8	2	1	4
PAL5-1-7	pAL5-1	Fl-5 (Lpe)	WF1-WR1	3.9	4	2	4
PAL5-1-9	pAL5-1	Fl-5 (Lpe)	WF1-WR1	3.9	3	1	4

^a Type 0 = no visible reaction, 1 = brown pinpoint spots, 2 = small brown lesions, 3 = typical blast lesions with intermediate sizes, 4 = typical blast lesions with large sizes, and 5 = complete blighting of leaf blades.

^b Isolate from which the template DNA was extracted (original host of the isolate; Sit = *Setaria italica*, Pmi = *Panicum miliaceum*, Eco = *Eleusine coracana*, Tae = *Triticum aestivum*, Asa = *Avena sativa*, and Lpe = *Lolium perenne*).

^c Primers used for amplification of the insert.

trol transformant were virulent on both CO39 (*Pi-CO39(t)*) and Yashiomochi (*pi-CO39(t)*) (Fig. 5). By contrast, PAS1-3-1 was avirulent on CO39 without losing virulence on Yashiomochi (Fig. 5). All the other transformants showed the same pattern of reactions on the two cultivars (Table 3). These results indicate that all the *AVR1-CO39* homologs tested encode the function of an avirulence gene recognized by CO39.

Distribution of *Pi-CO39(t)* in *Oryza* populations.

The finding that all *Oryza* isolates have lost the function of *AVR1-CO39* led to the hypothesis that the corresponding resistance gene *Pi-CO39(t)* is widely distributed among their host species, *Oryza* spp. To test this hypothesis, 65 cultivars or accessions of *Oryza* spp. were inoculated with PO12-7301-2 and its transformant carrying *AVR1-CO39* (PAS1-3-1). *AVR1-CO39* operates only when a plant cultivar carries its corresponding resistance gene, *Pi-CO39(t)*. Therefore, if a cultivar is susceptible to PO12-7301-2 and resistant to the transformant with *AVR1-CO39*, we can conclude that the cultivar carries *Pi-CO39(t)*. Among the 65 cultivars or accessions, 33 were susceptible to PO12-7301-2. Unexpectedly, all of them except CO39 also were susceptible to PAS1-3-1 (Table 4). This result indicates that *Pi-CO39(t)* is rare in these 33 cultivar or acces-

sions of *Oryza* spp. Due to epitasis of resistance genes, we cannot conclude whether the lines resistant to PO12-7301-2 carry the *Pi-CO39(t)*. Interestingly, all 65 of the rice lines tested were resistant to the *Setaria* isolate (GFSI1-7-2) and the *Triticum* isolate (Br48) that carry a functional *AVR1-CO39* homolog (Table 4).

DISCUSSION

Several avirulence genes have been cloned from the blast fungus (e.g., *PWL2* [Sweigard et al. 1995], *PWL1* [Kang et al. 1995], *AVR-Pita* [Orbach et al. 2000], and *ACE1* [Böhnert et al. 2004]). Most of them are distributed widely in *Pyricularia* isolates (Kang et al. 1995), but their evolutionary significance is unclear. The present study provided new insight into the process of evolution of an avirulence gene, *AVR1-CO39*. When the presence or absence of *AVR1-CO39* was plotted on a dendrogram constructed from rDNA sequences, a perfect parallelism was observed between its distribution and the phylogeny of *Pyricularia* isolates (Fig. 2), suggesting that the functional *AVR1-CO39* appeared during the early stages of evolution of a species, *P. oryzae*, and was lost during the early stages of evolution of the *Oryza*-specific subgroup of *P. oryzae*. Couch and

Fig. 4. Southern hybridization analysis of transformants (PAS1-3-1 through PAS1-3-15) of a rice isolate, PO-12-7301-2, with pAS1-3 that carries a 3.8-kb fragment containing an *AVR1-CO39* homolog derived from a *Setaria* isolate, GFSI1-7-2. **A**, Detection of the 3.8-kb fragment (arrowhead). Genomic DNA was double digested with *XhoI* and *NotI*, and hybridized with the 3' half (500 bp) of the 1.05-kb *AVR1-CO39* fragment derived from 2539. **B**, Determination of copy numbers of the transgene. Genomic DNA was double digested with *XhoI* and *EcoRV*, and hybridized with the 3' half fragment.

associates (2005) examined the distribution of *AVR1-CO39* in *M. oryzae* and found some *Oryza* isolates carrying *AVR1-CO39*. However, they are clustered with *Eleusine* isolates or *Panicum* isolates in their dendrogram and, therefore, may be *Eleusine* or *Panicum* isolates that colonized rice plants through opportunistic infection. Pathogenicity tests are needed to determine their primary hosts.

What is the significance of *AVR1-CO39*? If the rice cv. CO39 is attacked by *P. oryzae* isolates other than *Oryza* isolates, the *Pi-CO39(t)* gene product would recognize the *AVR1-CO39* gene product from these isolates and induce resistance reactions in rice plant tissues. In this case, *AVR1-CO39* undoubtedly is involved in the avirulence at the host species level (avirulence of non-*Oryza* isolates to a rice cultivar). However, it is unlikely that *AVR1-CO39* is involved generally in the host species specificity (avirulence of non-*Oryza* isolates on *Oryza* spp.) because its corresponding resistance gene, *Pi-CO39(t)*, is not widely distributed in the populations of *Oryza* spp. (Table 4). In fact, all the *Oryza* cultivars or accessions tested were resistant to a *Setaria* isolate (GFS11-7-2) and a *Triticum* isolates (Br48) even though they did not carry *Pi-CO39(t)* (Table 4). Furthermore, a genetic study demonstrated that the avirulence of Br48 on rice is a complex trait (data not shown), as has been previously suggested in crosses with a weeping lovegrass pathogen, 4091-5-8 (Valent et al. 1991). Undoubtedly, the functional *AVR1-CO39* homolog found in Br48 cannot fully explain the inability of this isolate to grow on rice.

Pi-CO39(t) is not only rare in the populations of *Oryza* spp. but also seems to be very rare or absent in the populations of *Setaria*, *Panicum*, *Eleusine*, *Avena*, *Triticum*, and *Lolium* spp. on the basis of information from literature. For example, Kato and associates (2000) reported that all *Eleusine* accessions

tested were susceptible to all *Eleusine* isolates tested. Their test isolates included G10-1, an *Eleusine* isolate employed in the present study. If the *Eleusine* accessions would contain *Pi-CO39(t)*, they should have been resistant to G10-1 because G10-1 carries *AVR1-CO39* (Fig. 2). Similar reasoning is applicable to cultivars or accessions of *Panicum* (Kato et al. 2000), *Setaria*, *Avena*, *Lolium* (Kato 1983), and *Triticum* (unpublished data). Therefore, there seems to be little opportunity for the products of *AVR1-CO39* and *Pi-CO39(t)* to meet in nature and elicit disease resistance. Nevertheless, all the *AVR1-CO39* homologs tested have maintained their function (Table 3). This result suggests that there may be a selection pressure for maintaining their integrity, which could be associated with “genuine” functions of avirulence genes, such as pathogenicity or fitness, as has been suggested in other systems (Knogge 1996; Ritter and Dangel 1995; Vera Cruz et al. 2000).

If the genuine function of *AVR1-CO39* is so important, why did *Oryza* isolates lose its function? One hypothesis is that a mutant strain or group with the rearrangements already had been present in the original *P. oryzae* population, and happened to gain the virulence on *Oryza* plants, resulting in an ancestor of the *Oryza*-specific subgroup. This hypothesis presumes that there is no relation of cause and effect between the loss of the *AVR1-CO39* function and the gain of the virulence on *Oryza* plants. However, the breakpoints and overall organizations of the two types of rearrangements, J and G types, are different, suggesting that they may have arisen by independent deletion events (Farman et al. 2002). It seems unlikely that two different mutants with independent rearrangements at the *Avr1-CO39* locus, both of which should have been very rare in the original *P. oryzae* population, gained virulence on the same plant genus *Oryza* by chance and converged into the *Oryza*-specific subgroup. It seems

Fig. 5. Pathogenicity of an *Oryza* isolate (PO-12-7301-2) and its transformants PBEv (carrying the pBluescript empty vector) and PAS1-3-1 (carrying the *AVR1-CO39* homolog derived from a *Setaria* isolate) on rice cvs. CO39 and Yashinomochi 7 days after inoculation.

more probable that there has been strong selection for loss of avirulence conferred by *AVR1-CO39*, as has been suggested by Farman and associates (2002).

A question then arises: how did *Pi-CO39(t)*, which is infrequent in the populations of *Oryza* spp. at present, confer such a strong selection pressure to the ancestral *Oryza* isolate or isolates? A clue may be found in the time lag between the evolution of host plants and the differentiation of the host-specific subgroups. It is apparent that *P. oryzae* has not co-evolved with gramineous plants because there is no parallelism between the phylogenetic relationship among the host-specific subgroups and that of their host plants (Kato et al. 2000). In the timescale of the differentiation of gramineous hosts, the differentiation of the host-specific subgroups should have been a recent event. Kato and associates (2000) pointed out that *Eleusine* isolates seem to maintain characteristics of an ancestral type of *P. oryzae* (e.g., the highest fertility compared with any other isolates of *P. oryzae*) and that the centers of domestication of foxtail millet, common millet, and rice are on the route of migration of finger millet from East Africa to Far East Asia. Based on these facts, they suggested that *P. oryzae* differentiated the host-specific subgroups on foxtail millet, common millet, and rice during the coincident migration with finger millet to Far East Asia. Their important conclusion is that the host-specific subgroups of *P. oryzae* have developed as a result of human migration and the dawn of agriculture within the last 10,000 years (Kato et al. 2000). We agree with their idea that the acquisition of the virulence on rice occurred recently in a confined area. We hypothesize that indigenous rice strains or populations in that area carried *Pi-CO39(t)* at a high frequency and, therefore, that *P. oryzae* was forced to lose *AVR1-CO39* corresponding to *Pi-CO39(t)* to parasitize rice plants. After fixation of the rearranged structures at the *AVR1-CO39* locus in the domestic rice-pathogenic population, the rice-pathogenic individuals may have spread all over the world.

Alternatively, *Pi-CO39(t)* once may have been widely distributed in *Oryza* spp., which provided the selection pressure for losing the function of *AVR1-CO39* in multiple locations in the world and resulted in the convergent evolution of *Oryza* isolates. Thereafter, the frequency of *Pi-CO39(t)* in *Oryza* spp. may have decreased gradually because this resistance gene was no longer useful. However, most of the present *Oryza* isolates from various locations in the world have either of the two nonfunctional allele types at the *Avr1-CO39* locus (Farman et al. 2002). The complexity of the changes found in the J- and G-type *Avr1-CO39* loci indicate that these events should not have occurred repeatedly in evolution (Farman et al. 2002). It therefore is unlikely that *Oryza* isolates originated from convergent evolution at multiple locations.

Interestingly, the two nonfunctional allele types at the *Avr1-CO39* locus (the G and J types) were congruous to the two major lineages previously defined for Japanese *Oryza* isolates using the repeat sequences MAGGY and MGR583 (Table 2). This may seem curious because extensive fingerprinting studies in the past decade have identified many unique lineages in each geographic region (Chen et al. 1995; Don et al. 1999b; Kumar et al. 1999; Levy et al. 1993; Roumen et al. 1997; Xia et al. 1993; Zeigler et al. 1995). In all, 5 to 10 were identified in Vietnam (Don et al. 1999b), Columbia (Levy et al. 1993), Europe (Roumen et al. 1997), the U.S.A. (Xia et al. 1993), and the Philippines (Chen et al. 1995; Zeigler et al. 1995), and more than 50 in the Indian Himalayas (Kumar et al. 1999). In most of these studies, however, a similarity level of 0.8 has been used to define lineage groups. Although Don and associates defined JL1 and JL2 at a level of 0.7, these lineages could be differenti-

ated even at a level of 0.6 with both MAGGY and MGR586 probes (Don et al. 1999a). If this similarity level is applied to the populations in the other countries, the number of lineages can be reduced to two or three. For example, the five lineages found in Vietnam are clustered into a large group (consisting of four lineages, VL2, VL3, VL4, and VL5) and a small group (consisting of only one lineage, VL1) at the similarity level of 0.6 (Don et al. 1999b). The Japanese isolates collected before 1960 were more diverse than those collected after 1970, but could be grouped into two fundamental groups at the level of 0.6 as mentioned above. This grouping was congruous to the allele types at the *Avr1-CO39* locus (Table 2). We assume that the two groups recognized at the level of 0.6, one including JL1 and the other including JL2, may be superlineages within which nest the other lineages. Don and associates (1999a) suggested that the genetic diversity in the rice blast population in Japan has decreased during the 1960s, probably due to the extensive cultivation of a limited number of cultivars with the same genotypes or successive breakdown of resistance genes. This simplification of the population structure of Japanese isolates may have made it easier to detect the superlineages. The origin of the exceptional isolate (P-2b) is not clear. This is an old isolate subcultured from 1948, and has a characteristic conidial morphology. Some rearrangements or mutations may have occurred in its genome.

Because the superlineages and the allele types at the *Avr1-CO39* locus are correlated, alterations that define the alleles must predate the origin of the lineages that are now found in various countries. Interestingly, both of these allele types are found at least in Japan and Vietnam (Fig. 2). In Japan, the G-type carriers belong to the group including the larger lineage JL1, whereas the J-type carriers belong to the other group, including the smaller lineage JL2 (Table 2). In Vietnam, the G-type carriers belong to the larger group consisting of the four lineages, whereas the J-type carrier belongs to the smaller group consisting of a single lineage (data not shown). Taken together, we assume that both groups were established in a confined area as superlineages, spread to other geographic zones, and then developed unique lineages within different geographical settings.

MATERIALS AND METHODS

Fungal materials.

Pyricularia strains used were composed of a laboratory strain, 2539, from which the 1.05-kb-containing *AVR1-CO39* fragment was first isolated, and 82 isolates collected from various gramineous plants (Table 1). These isolates have been verified in independent infection assays to be pathogenic on the host from which they originally were isolated. The strains were maintained in the Laboratory of Plant Pathology, Kobe University, at 4°C as dried-down cultures in barley seed media (Hayashi and Kato 1988), transferred to a potato dextrose agar slant just before use, and grown at room temperature. Cultures 1 week to 3 months old were used for experiments.

DNA extraction, Southern hybridization, and PCR screening.

Mycelial plugs were transferred to 50 ml of complete medium (CM) (yeast extract, 3 g; Casamino acid, 3 g; and sucrose, 5 g per liter) and grown at 25°C for 4 days. Total DNA was extracted from the resulting mycelia as described previously (Nakayashiki et al. 1999).

The 3' half fragment of *AVR1-CO39* was amplified from the genomic DNA of 2539 with primers AVF2 (5'-TCTACCTCTCTGCCACCATTTTACTTTTC-3') and AVR1-2 (5'-GC

GAATCCATAGACAAGGAC-3') (Fig. 1) in a 20- μ l reaction containing 0.5 unit of HotStarTaq DNA polymerase (QIAGEN, Hilden, Germany), 1 $\times$ PCR buffer provided by the manufacturer, 200 μ M each dNTP, 0.2 μ M each primer, and 10 ng of the template DNA. The reaction was heated to 95°C for 15 min and then amplified for 30 cycles (0.5 min at 94°C, 0.5 min at 55°C, and 1 min at 72°C), with the final step at 72°C for 10 min. The PCR product was electrophoresed in a 0.7% agarose gel and stained with ethidium bromide. A DNA band of the expected size (500 bp) was cut from the gel, purified with Min Elute gel extraction kit (QIAGEN), and labeled following the instructions of the ECL direct nucleic acid labeling and detection system (Amersham Pharmacia Biotech, Buckinghamshire, England).

Total DNA was digested with restriction enzymes (Takara, Tokyo) and electrophoresed on a 0.8% agarose gel (Nacalai Tesque, Kyoto, Japan) in 0.5 $\times$ Tris-borate-EDTA buffer overnight. The fractionated DNA was transferred to a nylon membrane (Hybond N+, Amersham Pharmacia Biotech) with 10 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)

and fixed by UV irradiation. The membrane was hybridized overnight with the labeled probe mentioned above in Gold hybridization buffer (Amersham Pharmacia Biotech) containing 0.5 M NaCl at 42°C. After hybridization, the membrane was washed twice in the primary wash buffer (0.4% sodium dodecyl sulfate and 0.5 $\times$ SSC) for 10 min at 55°C followed by two washes in 2 $\times$ SSC for 5 min at room temperature. Detection of target DNA was performed according to the instructions of the ECL Direct Nucleic Acid Labeling and Detection System.

PCR screening for *AVR1-CO39* homologs in the *Pyricularia* isolates was performed with primers AVF1-2 (5'-TGCC GCATTTTGCTAACC-3') and AVR1-2. A 20- μ l reaction containing the HotStarTaq DNA polymerase, the PCR buffer, dNTPs, the primers, and a template DNA at the concentration mentioned above was heated to 95°C for 15 min and then amplified for 30 cycles (0.5 min at 94°C, 0.5 min at 50°C, and 2 min at 72°C), with the final step at 72°C for 10 min. The PCR product was electrophoresed in a 0.8% agarose gel and stained with ethidium bromide.

Table 4. Survey of *Pi-CO39(t)* in *Oryza* populations using a transformant with *AVR1-CO39*

Population ^b	Provider ^c	Source ^c	Locality	Genome	Infection type with ^a			
					PO12-7301-2 (Osa) ^d	PAS1-3-1 ^e	GFSI1-7-2 (Sit) ^d	Br48 (Tae) ^d
<i>Oryza sativa</i> subsp. <i>japonica</i>								
Aichi-asahi	H. Kato	...	Japan	AA	3 (S)	3(S)	0	0
Kanto 51	H. Kato	...	Japan	AA	1	1	0	0
Fukunishiki	H. Kato	...	Japan	AA	2	1	0	0
Yashiromochi	H. Kato	...	Japan	AA	4 (S)	3 (S)	0	0
Toride 1	H. Kato	...	Japan	AA	3 (S)	3 (S)	0	0
Nipponbare	T. Ishii	...	Japan	AA	1	1	0	0
Taichung 65	T. Ishii	...	Taiwan	AA	0	0	0	0
Norin 22	T. Ishii	...	Japan	AA	2	2	0	1
501 (Urasan)	T. Ishii	NIGJ	Japan	AA	3 (S)	3 (S)	0	0
563 (Kinoshitamochi)	T. Ishii	NIGJ	Japan	AA	0	0	0	0
Yamadanishiki	C. Nakamura	...	Japan	AA	0	0	0	1
Reiho	C. Nakamura	...	Japan	AA	0	0	0	0
<i>O. sativa</i> subsp. <i>javanica</i>								
242	T. Ishii	NIGJ	Philippines	AA	3 (S)	4 (S)	0	0
532 (Gaisen)	T. Ishii	NIGJ	Japan	AA	4 (S)	3 (S)	0	0
647 (Padi ase banba)	T. Ishii	NIGJ	Philippines	AA	4 (S)	3 (S)	0	0
<i>O. sativa</i> subsp. <i>indica</i>								
Guichao 2	Y. Yamamoto	...	China	AA	0	0	0	0
BR3	Y. Yamamoto	...	Bangladesh	AA	0	0	0	0
AC130	Y. Yamamoto	...	Unknown	AA	0	0	0	0
Yanxuan 203	Y. Yamamoto	...	China	AA	0	0	0	1
Nanjing 11	Y. Yamamoto	...	China	AA	4 (S)	4 (S)	0	0
IR24	Y. Yamamoto	...	Philippines	AA	0	0	0	0
Tadukan	Y. Yamamoto	...	Philippines	AA	0	0	0	0
DAWN	Y. Yamamoto	...	America	AA	0	0	0	0
CO39	NIARJ	...	India	AA	4 (S)	1	0	1
IR36	T. Ishii	...	Philippines	AA	1	1	0	0
101	T. Ishii	NIGJ	Taiwan	AA	4 (S)	4 (S)	0	0
104	T. Ishii	NIGJ	Taiwan	AA	3 (S)	3 (S)	0	1
108 (Peiku)	T. Ishii	NIGJ	Taiwan	AA	0	0	0	0
115	T. Ishii	NIGJ	Taiwan	AA	3 (S)	3 (S)	0	1
144 (O luen chung)	T. Ishii	NIGJ	Taiwan	AA	4 (S)	4 (S)	0	0
414 (PTB10)	T. Ishii	NIGJ	India	AA	0	0	0	0
415	T. Ishii	NIGJ	India	AA	0	0	0	0
419 (PTB7)	T. Ishii	NIGJ	India	AA	0	0	0	1
435 (Pachchai perum)	T. Ishii	NIGJ	Sri Lanka	AA	4 (S)	4 (S)	0	0

(continued on next page)

^a Type 0 = no visible reaction, 1 = brown pinpoint spots, 2 = small brown lesions, 3 = typical blast lesions with intermediate sizes, 4 = typical blast lesions with large sizes, and 5 = complete blighting of leaf blades. Compatible interactions are marked by (S).

^b Plant species, cultivar or accession.

^c NIARJ = National Institute of Agrobiological Research of Japan, NIGJ = National Institute of Genetics of Japan, and IIRI = International Rice Research Institute.

^d Wild isolate (original host); Osa = *Oryza sativa*, Sit = *Setaria italica* and Tae = *Triticum aestivum*.

^e Transformant of PO-12-7301-2 with a homolog of *AVR1-CO39* derived from GFSI1-7-2.

^f Hybrid between *indica* and *japonica*.

Construction of a dendrogram based on rDNA sequences.

The ITS-1-5.8S-ITS2 region of the nuclear rDNA was amplified with the ITS4 and ITS5 primers (White et al. 1990) from total DNA of the *Pyricularia* isolates. A 20- μ l reaction containing the HotStarTaq DNA polymerase, the PCR buffer, dNTPs, the primers, and a template DNA at the concentration mentioned above was heated to 95°C for 15 min and then amplified for 30 cycles (1 min at 94°C, 1 min at 55°C, and 1 min at 72°C), with the final step at 72°C for 5 min. The PCR product was electrophoresed in a 0.8% agarose gel, cut from the gel, and purified with Min Elute gel extraction kit (QIAGEN). The purified DNA was sequenced directly with the ITS4 and ITS5 primers using the BigDye terminator cycle sequencing ready reaction kit and ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA, U.S.A.). Nucleotide sequences were aligned using the CLUSTAL W program (Thompson et al. 1994) and finally optimized manually. A phylogenetic tree was constructed using the neighbor-joining program (Saitou and Nei 1987) in PHYLIP v.3.4 (Felsenstein 1991). Evolutionary distances were calculated from the Kimura two-parameter method (Kimura 1980). An unrooted tree was rooted using an isolate from *Zingiber mioga* (HYZiM101-1-1-1)

as an outgroup. The robustness of clusters was assessed by bootstrap analysis (Felsenstein 1985).

PCR analysis of the rearrangement at the *Avr1-CO39* locus in *Oryza* isolates.

The types of rearrangements at the *Avr1-CO39* locus in *Oryza* isolates were determined by PCR as described previously (Farman et al. 2002) with a modification. The modification was that the P318 primer was replaced with the WR1 primer (5'-GTTTCTTGATCCCTTG-3') (Fig. 1) because P318 sometimes produced nonreproducible results.

Cloning and sequencing of *AVR1-CO39* homologs in *P. oryzae* isolates other than *Oryza* isolates.

A 3.9-kb fragment containing *AVR1-CO39* was amplified from genomic DNA of a representative isolate of each host-specific subgroup with primers WF1 (5'-AAGGGAAGAGTAAAGTAAATGTAGA-3) and WR1 (Fig. 1). A 50- μ l reaction containing 2.5 units of LA *Taq* (Takara), 1 $\times$ PCR buffer provided by the manufacturer, 200 μ M each dNTP, 0.2 μ M each primer, and 10 ng of the template DNA was heated to 95°C for 1 min and then amplified for 30 cycles (0.5 min at 94°C, 0.5 min at 55°C, and 4

Table 4. (continued from preceding page)

Population ^b	Provider ^c	Source ^c	Locality	Genome	Infection type with ^a			
					PO12-7301-2 (Osa) ^d	PAS1-3-1 ^c	GFSH1-7-2 (Sit) ^d	Br48 (Tae) ^d
437	T. Ishii	NIGJ	India	AA	3 (S)	3 (S)	0	1
444	T. Ishii	NIGJ	India	AA	0	0	0	0
1034	T. Ishii	NIGJ	China	AA	4 (S)	4 (S)	0	0
C5444 (Paddy NP137)	T. Ishii	NIGJ	India	AA	0	0	0	0
C8005	T. Ishii	NIGJ	India	AA	0	0	0	1
f-151	Y. Yamamoto	...	Bangladesh	AA	3 (S)	3 (S)	0	0
<i>O. sativa</i> ^f								
Suweon 258	Y. Yamamoto	...	Korea	AA	0	0	0	0
Milyang 23	Y. Yamamoto	...	Korea	AA	0	0	0	0
<i>O. glaberrima</i>								
W401 (Ali N'Gone)	T. Ishii	NIGJ	Guinea	AA	2	2	2	0
W438 (Kebele)	T. Ishii	NIGJ	Unknown	AA	0	0	1	1
W440 (Hashitalmi)	T. Ishii	NIGJ	Guinea	AA	2	2	2	1
W446 (Konga)	T. Ishii	NIGJ	Guinea	AA	2	2	2	1
W492 (Maro Tiede)	T. Ishii	NIGJ	Unknown	AA	3 (S)	3 (S)	2	2
W528 (Kondou)	T. Ishii	NIGJ	Guinea	AA	1	1	2	0
<i>O. rufipogon</i>								
W630	T. Ishii	NIGJ	Myanmar	AA	3 (S)	3 (S)	0	0
W1956	T. Ishii	NIGJ	China	AA	2	2	0	0
<i>O. glumaepatula</i>								
W1186	T. Ishii	NIGJ	Surinam	AA	4 (S)	4 (S)	0	0
<i>O. meridionalis</i>								
W1299	T. Ishii	NIGJ	Australia	AA	4 (S)	4 (S)	0	0
W1300	T. Ishii	NIGJ	Australia	AA	3 (S)	3 (S)	0	0
W1629	T. Ishii	NIGJ	Australia	AA	4 (S)	4 (S)	0	0
<i>O. meridionalis</i>								
W1633	T. Ishii	NIGJ	Australia	AA	0	0	0	0
W1638	T. Ishii	NIGJ	Australia	AA	3 (S)	3 (S)	0	0
<i>O. barthii</i>								
W607	T. Ishii	NIGJ	Guinea	AA	4 (S)	4 (S)	0	1
W720	T. Ishii	NIGJ	Sudan	AA	4 (S)	4 (S)	1	1
W825	T. Ishii	NIGJ	Mali	AA	4 (S)	4 (S)	1	1
W1152	T. Ishii	NIGJ	Unknown	AA	4 (S)	4 (S)	1	1
<i>O. officinalis</i>								
100896	T. Ishii	IRRI	Tailand	CC	3 (S)	3 (S)	0	0
<i>O. latifolia</i>								
100914	T. Ishii	IRRI	Mexico	CCDD	4 (S)	4 (S)	0	0
103787	T. Ishii	IRRI	Colombia	CCDD	4 (S)	4 (S)	0	0
<i>O. australiensis</i>								
101410	T. Ishii	IRRI	Australia	EE	4 (S)	4 (S)	0	0
105267	T. Ishii	IRRI	Australia	EE	4 (S)	4 (S)	0	0
<i>Setaria italica</i>								
Beniawa	H. Kato	...	Japan	...	1	1	5 (S)	2
<i>Triticum aestivum</i>								
Norin 4	K. Tsunewaki	...	Japan	...	1	1	1	5 (S)

min at 72°C), with the final step at 72°C for 10 min. The PCR product was electrophoresed in a 0.9% agarose gel in 1× Tris-acetate-EDTA buffer and stained with ethidium bromide. When the amplification was unstable, the WF1 primer was replaced with the WF9 primer (5'-GCAGCTACATCCAATCATTA-3') (Fig. 1). The expected size of the amplicon from this primer combination was 3.8 kb. When amplicons were not obtained with the WF1-WR1 or WF9-WR1 combinations, the target region was narrowed to a 1.7-kb fragment flanked by WF6 (5'-TAAACACCGCCTGAGATAGT-3') and WR4 (5'-TGGTCACG GTTGGCAGTC-3') (Fig. 1).

A PCR product with the expected size was cut from the gel, purified with Min Elute gel extraction kit (QIAGEN), and then incubated with 2 units of T4 polymerase (Takara) at 37°C for 10 min. The blunt-ended fragment was inserted into the *EcoRV* site of BluescriptII SK(+) using DNA ligation kit ver.2 (Takara). The ligation product was transformed into *Escherichia coli* XL1-Blue, and three colonies were recovered from each ligation reaction. The recombinant plasmid was extracted from each of the three cultures (colonies) with QIAprep spin miniprep kit (QIAGEN), and sequenced by primer walking using the BigDye Terminator Cycle Sequencing Ready Reaction Kit and ABI Prism 310 genetic analyzer (Applied Biosystems). The authentic DNA sequence in the genomic DNA was determined by comparing the sequences of the three clones derived from the three colonies.

Transformation of *P. oryzae*.

Of the three clones containing the *AVR1-CO39* homolog derived from each *P. oryzae* isolate, a clone was chosen whose DNA sequence at the *AVR1-CO39* 1.05-kb fragment was identical to the inferred authentic sequence in the genomic DNA. In all, eight plasmids (Table 3, pAX7-17 through pAL5-1) were employed for co-transformation of PO-12-7301-2, an *Oryza* isolate of *P. oryzae*, with pSH75 containing the hygromycin B phosphotransferase gene (Kimura and Tsuge 1993).

Mycelial plugs of PO-12-7301-2 were transferred to 50 ml of CM broth and grown at 25°C in the dark. Three-day-old cultures were homogenized briefly at full speed in a blender, transferred to 100 ml of fresh CM broth, and further incubated at 28°C for 24 h. The mycelia were harvested by filtration through two layers of gauze, washed with OM buffer (10 mM Na₂HPO₄, 1.2 M MgSO₄, pH 5.8), suspended in 50 ml of filter-sterilized enzyme solution (lysing enzymes [Sigma-Aldrich Japan, Tokyo] at 10 mg/ml in OM buffer), and incubated at 25°C for 3 h with vigorous shaking. The protoplast suspension was mixed with two volumes of STC solution (20% sucrose, 25 mM Tris-HCl, pH 7.5, 25 mM CaCl₂) and centrifuged at 800 × *g* for 10 min. The pellet was washed twice with 3 to 5 ml of STC solution and finally suspended in STC solution at 1 × 10⁸ protoplasts/ml.

An aliquot (150 µl) of the protoplast suspension was mixed with a plasmid containing an *AVR1-CO39* homolog and pSH75 (3 µg each), and incubated on ice for 10 min. The suspension then was mixed with 200, 400, and 800 µl of PEG solution (60% polyethylene glycol 3350 in 25 mM Tris-HCl, pH 8.0, and 25 mM CaCl₂) in a step-by-step manner, and incubated again on ice for 10 min. PEG solution was removed by centrifugation at 2,000 × *g* for 5 min. Collected protoplasts were suspended in 5 ml of regeneration medium (CM containing 20% wt/vol sucrose) and incubated overnight at room temperature. The protoplast suspension then was mixed with 40 ml of regeneration agar medium (CM containing 20% wt/vol sucrose and 1.5% agarose) that had been melted and kept at 50°C, and plated into petri dishes. After incubation at 26°C for 16 h, selective agar medium (CM containing 0.7% agarose and hygromycin B at 400 µg/ml) was overlaid. After incubation at 26°C for 6 to 7 days, regenerants that appeared on the selective

agar medium were transferred to PDA media containing hygromycin B at 400 µg/ml for secondary screening.

Pathogenicity assay.

Seed of *Oryza* spp. (5 seeds/cultivar) were sown in soil in plastic pots (26 by 18 by 7 cm) and grown in a controlled-environment room with natural light at 25°C for 15 to 20 days. Seed of *Triticum aestivum* cv. Norin 4 and *Setaria italica* cv. Beniawa (5 seeds/cultivar) were sown in soil in plastic pots (15 by 6 by 10 cm) and grown in a controlled-environment room with fluorescent lighting (150 µmol s⁻¹ m⁻²) at 25°C for 7 days. Conidial suspension (2 × 10⁵/ml) prepared as described previously (Murakami et al. 2000) was sprayed on the seedlings with 0.01% Tween 20. Inoculated seedlings were incubated in a dark, moistened box for 24 h at 25°C and then returned to the controlled-environment room with fluorescent lighting mentioned above. At 6 to 7 days after inoculation, infection was evaluated using six scores from 0 to 5, where 0 = no visible reaction, 1 = brown pinpoint spots, 2 = small brown lesions, 3 = typical blast lesions with intermediate sizes, 4 = typical blast lesions with large sizes, and 5 = complete blighting of leaf blades. The types 0, 1, and 2 were regarded as avirulent or resistant reactions whereas the types 3, 4, and 5 were considered virulent or susceptible reactions. All inoculation tests were repeated at least three times.

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