Genetic Diversity of *avrBs*-like Genes in Three Different *Xanthomonas* Species Isolated in Korea

Chang-Sik Oh^{1,3}, Seungdon Lee² and Sunggi Heu^{1*}

¹Microbial Safety Division, National Academy of Agricultural Science, RDA, Suwon 441-707, Korea

²Research Coordination Division, RDA, Suwon 441-707, Korea

³Department of Horticultural Biotechnology, Kyung Hee University, Yong-In 446-701, Korea

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Plant-pathogenic bacteria including *Xanthomonas* spp. carry genetic diversity in composition of avirulence genes for interaction with their host plants. Previously, we reported genetic diversity of avirulence genes in *X. axonopodis* pv. *glycines*. In this study, we determined genetic diversity of five avirulence genes, *avrBs1*, *avrBs2*, *avrBs3*, *avrBs4*, and *avrRxv*, in three other *Xanthomonas* species isolated in Korea by genomic southern hybridization. Although Korean races of *X. campestris* pv. *vesicatoria* that were isolated from year 1995 to 2002 had the same avirulence gene patterns as those that already reported, there was race shift from race 3 to race 1 by acquisition of *avrBs3* genes. *X. campestris* pv. *campestris* isolated from Chinese cabbage, but not from cabbage or radish, carried two *avrBs3* genes, and one of them affected HR-eliciting ability of this bacterium in broccoli. *X. oryzae* pv. *oryzae* carried eight to thirteen *avrBs3* gene homologs, and this bacterium showed dynamic changes of resistance patterns in rice probably by losing or obtaining *avrBs3* genes. These results indicate that *avrBs3* gene is more diverse in *Xanthomonas* spp. than other four avirulence genes and also host ranges of these bacteria can be easily changed by loss or acquisition of *avrBs3* genes.

Keywords : *avrBs3*, *Xanthomonas campestris* pv. *campestris*, *Xanthomonas oryzae* pv. *oryzae*, host range

Gram-negative plant pathogenic bacteria, including *Xanthomonas* species, deliver many effector proteins into plant cells through the type III secretion system (T3SS) (Alfano and Collmer, 2004). Those effector proteins have been shown to manipulate host defense signaling pathways, by targeting and interfering with certain plant proteins. One group of bacterial effector proteins is transcription activator-like (TAL) effectors. They act like plant transcription factors and directly control expression of plant genes by

targeting to the plant nucleus (Kay et al., 2007). The best well-characterized TAL effector is AvrBs3 protein from *X. campestris* pv. *vesicatoria*, causing bacterial spot disease on tomato and pepper (Schornack et al., 2006).

The *avrBs3* gene in *X. campestris* pv. *vesicatoria* originally cloned from strain 71-21 is a single copy gene that encodes a protein with three distinct domains (Bonas et al., 1989). AvrBs3 protein has two nuclear localization signals and transcriptional activation domain in its C-terminus (Van den Ackerveken et al., 1996). It also has 17.5 times of 34 amino acid repeats in its central region. This repeat region has been shown to be critical for protein dimerization and DNA binding activity of AvrBs3 (Schornack et al., 2006). AvrBs3 protein causes hypertrophy of plant mesophyll cells that may help bacterial release to the plant leaf surface in the late stage of infection. Hypertrophy by AvrBs3 protein results from induced expression of certain plant genes with UPA (upregulated by AvrBs3) box in their promoters such as *UPA20* gene (Kay et al., 2007). The resistance pepper cultivars to *X. campestris* pv. *vesicatoria* strains carrying *avrBs3* gene contain a corresponding resistance gene, *Bs3*. This gene encodes a putative flavin monooxygenase, and its expression is controlled by the promoter with UPA box (Romer et al., 2007). The function of *Bs3* protein is not clear, but *Bs3*-mediated resistance is triggered by recognition of UPA box by AvrBs3, resulting in induction of the hypersensitive response (HR). *X. campestris* pv. *vesicatoria* has a AvrBs3 homolog, AvrBs4, which has very similar domain structures and is recognized by *Bs4* resistance protein (Schornack et al., 2004).

Other *Xanthomonas* species such as *X. oryzae* and *X. axonopodis* have been shown to contain *avrBs3* gene homologs (Kay and Bonas, 2009; Schornack et al., 2006). In particular, *X. oryzae* pv. *oryzae* causing bacterial leaf blight on rice and *X. axonopodis* pv. *citri* causing bacterial canker on citrus plants have multiple AvrBs3 homologs. Among them, AvrXa7 and AvrXa27 of *X. oryzae* pv. *oryzae* and PthA of *X. axonopodis* pv. *citri* have been relatively well characterized, and all contribute to bacterial pathogeni-

*Corresponding author.

Phone) +82-31-290-0420, FAX) +82-31-290-0406

E-mail) heu@rda.go.kr

city on each host plant (Gu et al., 2005; Swarup et al., 1992; Yang and White, 2004; Yang et al., 2000). Previously, we reported that *X. axonopodis* pv. *glycines* strains causing bacterial pustule disease on soybean carry three to seven *avrBs3* gene homologs, based on genomic southern hybridization with 155 strains isolated in Korea (Park et al., 2008). We also reported that *X. axonopodis* pv. *glycines* strains carry a single copy of *avrBs2* gene, but neither *avrBs1* nor *avrRxv*.

X. campestris pv. *vesicatoria* has been shown to carry more than 15 genes, including *avrBs1*, *avrBs2*, and *avrRxv*, that encode effector proteins (Kay and Bonas, 2009). *avrBs1* and *avrBs2* genes are critical avirulence genes to determine races of *X. campestris* pv. *vesicatoria*, along with *avrBs3* gene. Initially, pepper races of this bacterium were determined based on HR induction in pepper ECW differential cultivars carrying *Bs1*, *Bs2*, or *Bs3* resistance genes (Jones and Stall, 1998). For example, while pepper race 1 including the strain 71-21 induces the HR in both ECW-20R and ECW-30R because it carries both *avrBs2* and *avrBs3* genes, pepper race 2 induces the HR in both ECW-10R and ECW-20R because it carries both *avrBs1* and *avrBs2* genes. Pepper race 3 induces the HR only in ECW-20R because it carries only *avrBs2* gene. While the function of AvrBs1 protein is still unknown, AvrBs2 protein is a putative glycerophosphoryldiester phosphodiesterase (Swords et al., 1996). AvrRxv that encodes a putative cysteine protease or acetyltransferase belongs to YopJ/AvrRxv protein family (Ciesiolka et al., 1999).

In this study, we examined genetic diversity of five avirulence genes, *avrBs1*, *avrBs2*, *avrBs3*, *avrBs4*, and *avrRxv* in three *Xanthomonas* species isolated in Korea by genomic southern hybridization. We show race shift by uptake of *avrBs3* genes in *X. campestris* pv. *vesicatoria* and *X. oryzae* pv. *oryzae* within a short time period. In addition, we show that *X. campestris* pv. *campestris* strains isolated from Chinese cabbage carry two *avrBs3* genes, and one of them affects recognition of this bacterium in broccoli.

Materials and Methods

Bacterial strains. Bacterial strains of *X. campestris* pv. *vesicatoria* and *X. oryzae* pv. *oryzae* were isolated from pepper and rice, respectively, grown in Korea during year 1995 to 2003. *X. campestris* pv. *campestris* strains were isolated from Chinese cabbage, cabbage or radish grown in Korea during the same years. All strains collected were first tested for HR using nonhost plant, tobacco. Identities of all strains were confirmed by determining compositions of fatty acid methyl esters (FAME) with gas chromatography and also by using Biolog identification system (Bio-Rad, CA, USA), according to the manufacturer's instructions.

Bacterial strains were preserved in at -80°C for long-term storage, retrieved from the stock and were not transferred more than 2 or 3 times before testing pathogenicity.

Xanthomonas spp. were grown in peptone-sucrose agar (PSA) for 2 days at 28°C and *Escherichia coli* was grown in LB medium at 37°C . The concentrations of antibiotics incorporated into media were as follows: rifampicin, 50 ug/ml; kanamycin, 50 ug/ml; tetracycline, 15 ug/ml; ampicillin, 50 ug/ml.

Genomic southern hybridization. Southern hybridization was performed as described previously (Park et al., 2008). Briefly, total genomic DNA digested with *Bam*HI was electrophoresed in 0.7% agarose gel, depurinated with 0.25 N HCl for 15 min and transferred onto nylon membrane (GE Healthcare, NJ, USA) using vacuum blotter (BioRad). The transferred DNA was cross linked on the membrane with UV for 3 min. The dried membrane was prehybridized with hybridization solution [5X SSC, N-laurylsarcosine, 0.1% (W/V); SDS, 0.2% (W/V); Blocking reagent, 1% (W/V)] at 68°C for 1 hr. Hybridization was performed with fresh 10 ml hybridization solution with denatured gene probes for 100 cm² of membrane at 68°C for 16 hrs.

For the preparation of the probes for *avrBs1*, *avrBs2*, and *avrRxv*, DNA fragments of 1.2 kb, 2.1 kb, and 1.1 kb, respectively, were amplified and cloned into pTOPO cloning vector by using the following primer pairs: BS1F (5'-ATGTCCGACATGAAAGTTAATTTC-3') and BS1R (5'-TCTCCTGCATTTGTAACATG-3') for *avrBs1*; BS2F (5'-CGCGTATAGGTCCTCCGCAAC-3') and BS2R (5'-CAATCCGGCTCGGTCTGGTTG-3') for *avrBs2*; RXVF (5'-TATGRGCGACTCCATAAGAG-3') and RXVR (5'-CTCAGCGCGCCGAGTCGCGCGG-3') for *avrRxv*. For the gene probe of both *avrBs3* and *avrBs4*, the plasmid pBSavrXa10 containing *avrXa10*, one of *avrBs3* gene homologs of *X. oryzae* in the pBluescript II KS+ vector was used (Hopkins et al., 1992). Each gene probe was labeled by using the Megaprime DNA labeling kit (GE Healthcare).

Mutagenesis of *avrBs3* gene homolog in *X. campestris* pv. *campestris*. To mutate *avrBs3* homolog in *X. campestris* pv. *campestris*, *avrXa10* in the plasmid, pBSavrXa10, was first mutated *in vitro* by using EZ::Tn Km transposon kit (EPICENTRE, WI, USA). The plasmid, in which EZ::Tn Km was inserted in *avrXa10* gene, was screened and confirmed by DNA sequencing. The resultant plasmid was transformed into *X. campestris* pv. *campestris* strain 1175 isolated from Chinese cabbage by Gene Pulser (Bio-Rad) for mutation of *avrBs3* gene homolog based on double crossover recombination. Putative mutants were selected on LB agar supplemented with rifampicin, kanamycin, and ampicillin. To verify that double crossover had occurred in

avrBs3 gene homolog *X. campestris* pv. *campestris* strain 1175, selected putative mutants were confirmed by genomic southern hybridization with *avrBs10* gene probe.

Disease and HR assays. For disease assay in crucifer plants, 10⁸ CFU/ml of *X. campestris* pv. *campestris* isolated from Chinese cabbage, cabbage, or radish was inoculated by spraying bacterial suspension. With rice monogenic lines, *X. oryzae* pv. *oryzae* strains were inoculated by cutting the top edge of leaves by scissors after dipping into bacterial suspension (10⁸ CFU/ml). Inoculated plants were kept at the greenhouse for 5 days for *X. campestris* pv. *campestris* and for 10 days for *X. oryzae* pv. *oryzae* and the disease severity was scored by the lesion length (cm). For HR assay, 10⁸ CFU/ml of *X. campestris* pv. *campestris* wild type and mutant strains was infiltrated in broccoli leaves by needleless syringe. HR was scored 24 hours after infiltration.

Results

Genetic diversity of five avirulence genes in *X. campestris* pv. *vesicatoria* strains isolated in Korea. Races of *X. campestris* pv. *vesicatoria* are determined, based on presence of *avrBs1*, *avrBs2*, or *avrBs3* genes, as described in Introduction. We first determined if this general pattern is conserved in strains isolated in Korea from year 1995 to 2002 by southern hybridization with gene probes of *avrBs1*, *avrBs2*, and *avrBs3* originated from the American strains 85-10 (pepper race 2) and 71-21 (pepper race 1). We also checked two more avirulence genes, *avrBs4* and *avrRxv*. Southern results showed that Korean race 1 of *X. campestris*

pv. *vesicatoria* carries four avirulence genes except *avrBs1*, and Korean race 3 carries three avirulence genes except *avrBs1* and *avrBs3*, as summarized in Fig. 1A. This pattern was the same as reported in American strains.

We further examined the presence of *avrBs3* gene and its homolog *avrBs4* in 15-20 individual strains from year 1995 to 2002. We grouped strains from year 1995 to 1998, year 1999 to 2000, and year 2001 to 2002. From this assay, we found that only a few strains from year 1995-1998 carried the *avrBs3* gene, but the number of strains dramatically increased during 2001-2002 (Fig. 1B). In contrast, distribution of the *avrBs4* gene was not changed significantly. These results indicate that there was race shift from race 3 to race 1 due to uptake of the *avrBs3* gene.

Genetic diversity of four avirulence genes in *X. campestris* pv. *campestris* strains isolated from three different crucifer plants in Korea. Previously it was shown that *X. campestris* pv. *campestris* carries *avrBs1*, *avrBs2*, and *avrRxv*, but not *avrBs3* gene (Kay and Bonas, 2009). We isolated 47 strains from three different crucifer plants: 27 from Chinese cabbage, 14 from cabbage, and 6 from radish. We examined the presence of four avirulence genes by southern hybridization in these strains. Southern results showed that *avrBs2* was present in all strains, but *avrRxv* was not (Fig. 2A). More interestingly, *avrBs1* was only present in strains isolated from cabbage, but not from Chinese cabbage or radish. In addition, *avrBs3* gene was present in strains only isolated from Chinese cabbage, but not from cabbage or radish, and two different copies were detected (Fig. 2A and 2B). These results indicate that *avrBs3* genes are present in

Fig. 1. Genetic diversity of five avirulence genes in *X. campestris* pv. *vesicatoria* (*Xcv*) isolated in Korea by genomic southern hybridization. (A) Summary of genomic southern hybridization with race 1 and race 3 by using gene probes of five avirulence genes indicated in the figure. + and – indicate the presence and absence of indicated genes, respectively. (B) Southern hybridization of 11-12 different strains isolated in each indicated year period, using gene probes of *avrBs3*. Since *avrBs3* and *avrBs4* share 97% sequence identity and only two bands were detected, the bigger band was indicated as an *avrBs4* homolog. * indicates strains that belong to race 1.

Fig. 2. Genetic diversity of four avirulence genes in *X. campestris* pv. *campestris* (*Xcc*) isolated from three crucifer plants in Korea by genomic southern hybridization. (A) Summary of genomic southern hybridization with 27 strains from Chinese cabbage, 14 strains from cabbage and 6 strains from radish. + and – indicate the presence and absence of indicated genes, respectively. (B) Southern hybridization of 4 representative strains isolated from each plant, using gene probes of *avrBs1* and *avrBs3*.

X. campestris pv. *campestris* strains originated from certain host plants, and *avrBs1* or *avrBs3* genes may affect host range of this bacterium.

To determine host range of *X. campestris* pv. *campestris* strains, we tested disease development in six different crucifer plants, as shown in Table 1. The strains isolated from radish were highly virulent only in radish roots. While the *avrBs3*-carrying strains isolated from Chinese cabbage were highly virulent in Chinese cabbage, cabbage, chingensai, and rae rape, the *avrBs1*-carrying strains isolated from cabbage were highly virulent in cabbage, chingensai and broccoli. These results indicate that *avrBs1* and *avrBs3* genes affect their host ranges.

An *avrBs3* gene in *X. campestris* pv. *campestris* strains isolated from Chinese cabbage acts like a host range

Table 1. Disease severity of crucifers infected by *X. campestris* pv. *campestris* (*Xcc*) isolated from different host plants

Crucifers	<i>Xcc</i> isolated from		
	Chinese cabbage	Cabbage	Radish
Chinese cabbage	+++++ ^a	++	+
Cabbage	+++++	+++++	–
Chingensai	+++++	+++++	+
Rae Rape	+++++	+	–
Broccoli	–	+++++	–
Radish (Leaf)	+	++	–
Radish (Root)	+	+	+++++

^a the amount of disease severity: +++++, highly virulent; ++ or +, marginally virulent; –, avirulent

Fig. 3. Analysis of Chinese cabbage strain of *X. campestris* pv. *campestris* mutated in one of two *avrBs3* gene homologs. (A) Southern hybridization of two wild type (WT) and two mutant strains using *avrBs3* gene probe. Arrow indicates the mutated gene of *avrBs3* homolog. (B) HR assay in broccoli using the wild type (red circles) and mutant strain (blue circles).

determinant. From disease assay shown in Table 1, we found that strains from cabbage were highly virulent, but strains from Chinese cabbage were completely avirulent in broccoli. To determine if *avrBs3* genes present in strains isolated from Chinese cabbage affect this phenotypic difference, we mutated one of two *avrBs3* genes in two strains isolated from Chinese cabbage, by using the *avrXa10::EZ::Tn*, as described in Materials and Methods. As shown in Fig. 3A, the bottom band of *avrBs3* genes was disappeared in mutant strains. We tested if this mutation affects HR induction in broccoli, compared to the wild type. While the wild type induced a strong HR in broccoli, the mutant did not (Fig. 3B), indicating that broccoli can recognize AvrBs3 of strains from Chinese cabbage and this recognition makes this strain avirulent in broccoli.

Genetic diversity of *avrBs3* genes in *X. oryzae* pv. *oryzae* strains isolated from rice in Korea. Previously it was shown that *X. oryzae* pv. *oryzae* carries *avrBs2* and *avrBs3*, but neither *avrBs1* nor *avrRxv* (Kay and Bonas, 2009). We isolated 78 strains from rice in Korea and examined if they carry those four genes by genomic southern hybridization. Interestingly, all strains carried only *avrBs3* genes (Fig. 4A), and the number of *avrBs3* genes varied eight to thirteen

Fig. 4. Genetic diversity of four avirulence genes in *X. oryzae* pv. *oryzae* (*Xoo*) isolated in Korea by genomic southern hybridization. (A) Summary of genomic southern hybridization with 78 strains isolated nationwide of Korea by using gene probes of four avirulence genes indicated in the figure. + and - indicate the presence and absence of indicated genes, respectively. (B) Southern hybridization of 18 different strains using *avrBs3* gene probe.

among strains (Fig. 4B). Previously, we reported that *X. axonopodis* pv. *glycines* strains have three to seven of *avrBs3* genes (Park et al., 2008). The variation of *avrBs3* genes in

X. oryzae pv. *oryzae* was higher than *X. axonopodis* pv. *glycines* and two other *Xanthomonas* bacteria described above.

Using nine different rice monogenic lines (Huang et al., 1997), we further determined disease resistance patterns of *X. oryzae* pv. *oryzae* strains isolated for four years (year 1999 to 2003). We found that resistance phenotype was broken down by appearance of new races of *X. oryzae* pv. *oryzae*, because resistance mediated by dominant resistance genes, Xa1, Xa3, Xa7, and Xa8, was overcome by some strains in year 2002, 2001, 2001, and 2003, respectively (Table 2). In addition, we also found that some strains obtained avirulence genes recognized by xa13 in year 2003. In contrast, resistance mediated by a recessive resistance gene, xa5, was not broken down by any strains tested in our study. These results indicate that *X. oryzae* pv. *oryzae* strains lose their own avirulence genes or obtain new avirulence genes within a short time period to modulate interaction with host plants.

Discussion

In this study, we have shown that *Xanthomonas* spp. carry dynamic genetic diversity in composition of avirulence genes, and diversity of *avrBs3* gene among or within species is very high. In addition, we have shown that *Xanthomonas*

Table 2. Responses of rice monogenic lines to *X. oryzae* pv. *oryzae* strains isolated in year 1999–2003

Year	Strains #	Rice monogenic lines								
		IRBB1 (Xa1)	IRBB3 (Xa3)	IRBB4 (Xa4)	IRBB5 (xa5)	IRBB7 (Xa7)	IRBB8 (Xa8)	IRBB10 (Xa10)	IRBB13 (xa13)	IRBB21 (Xa21)
1999	1917	R	R	S	R	R	R	S	S	R
	1918	R	R	R	R	R	R	S	S	S
	1919	R	R	R	R	R	R	S	S	R
	1920	R	R	S	R	R	R	S	S	R
2001	2122	R	S*	S	R	R	R	S	S	S
	2123	R	S*	S	R	R	R	S	S	S
	2124	R	S*	S	R	R	R	S	S	S
	2125	R	R	S	R	S*	R	S	S	R
2002	2235	R	R	R	R	R	R	S	S	S
	2236	R	S	S	R	S	R	S	S	S
	2237	R	R	S	R	S	R	S	S	S
	2238	S*	S	R	R	R	R	S	S	R
2003	2345	R	S	R	R	R	R	S	R**	S
	2346	R	R	S	R	R	R	S	R**	R
	2347	S	R	R	R	R	S*	S	S	R
	2348	R	S	S	R	S	R	S	R**	S

R, resistant; S, susceptible

*: appearance of susceptible strains, compared to the previous year

** : appearance of resistant strains, compared to the previous year

spp. can easily lose or obtain *avrBs3* genes and change their host ranges within a short time period. In particular, this characteristic was apparent in *X. oryzae* pv. *oryzae* because each strain of this bacterium carries different numbers of *avrBs3*-like genes and many of them carry both virulence and avirulence activities in host plants, as reported (Nino-Liu et al., 2006).

AvrBs3 and its homologs are highly homologous, but they have various numbers of 34 or 35 amino acids tandem repeats in their central regions, which have been shown to determine their specificity. Within 34 amino acids in each repeat, 12th and 13th amino acids are highly variable (Boch et al., 2009). Recently, two papers have showed that these two highly variable amino acids in each repeat determine target DNA base specificity and they bind to each base in UPA box that locates in the promoter region of genes regulated by AvrBs3 and its homologs (Boch et al., 2009; Moscou and Bogdanove, 2009). Based on southern hybridization, *X. oryzae* pv. *oryzae* carries eight to thirteen different *avrBs3* gene homologs, and they look like a ladder (Fig. 4B). This may indicate that these AvrBs3 homologs have various numbers of tandem repeats, and their DNA target specificity is very likely different. In addition, due to high homology among *avrBs3* gene homologs, they can easily recombine one another and duplicate or remove certain *avrBs3* genes. This could be plausible explanation of why *Xanthomonas* spp. can easily change their race patterns within a short time period.

X. campestris pv. *campestris* can infect various numbers of crucifer plants. So far, most of studies with this bacterium have been performed with strains isolated from cabbage. As reported previously (Kay and Bonas, 2009), *X. campestris* pv. *campestris* strains isolated from cabbage in Korea carry both *avrBs1* and *avrBs2* genes, but not *avrBs3* gene. However, strains isolated from Chinese cabbage carried *avrBs2* and *avrBs3*, but not *avrBs1*. Moreover, strains isolated from radish only carried *avrBs2* gene. These patterns are quite different from previous reports. More interestingly, based on disease assay on crucifer plants, either *avrBs1* or *avrBs3* must be present in *X. campestris* pv. *campestris* to cause disease on Chinese cabbage or cabbage because strains from radish can not infect either plant. This is consistent with notion that these two genes are necessary for virulence of *Xanthomonas* spp. (Gurlebeck et al., 2006). In addition, strains from Chinese cabbage were completely avirulent in broccoli, but strains from cabbage were highly virulent. This implies that broccoli has a recognition system for AvrBs3, but not for AvrBs1. Indeed, a mutant strain of *X. campestris* pv. *campestris* from Chinese cabbage can not induce the HR in broccoli, indicating that broccoli has a corresponding resistance protein like Bs3.

Rice and pepper are two major crops growing in Korea.

In our study, we found race shift in both *X. campestris* pv. *vesicatoria* and *X. oryzae* pv. *oryzae*. This indicates that high selection pressure for this shift is present in the Korean environment. Based on our study, race shift in *X. campestris* pv. *vesicatoria* was directional, i.e. race 3 to race 1 by acquiring *avrBs3* gene. Although many different cultivars of pepper have been bred and grown in Korea for a long time, comprehensive analysis to determine resistance patterns of those cultivars has not been done yet. However, directional race shift may indicate that many pepper cultivars growing in Korea at present do not contain *Bs3* gene, and that is why *X. campestris* pv. *vesicatoria* acquires *avrBs3* gene to increase its fitness on pepper plants (Leach et al., 2001). Unlike *X. campestris* pv. *vesicatoria*, *X. oryzae* pv. *oryzae* seems to remove (or mutate) certain *avrBs3* gene homologs to infect certain resistance cultivars because most of cases we found were appearance of new strains that can infect resistant cultivars. Interestingly, in one case, the rice cultivar carrying *xa13* gene turned resistant to some strains of *X. oryzae* pv. *oryzae*. This is a quite unusual pattern because those strains acquired a gene to decrease their fitness in certain cultivars, but it could be explainable if *avrXa13* gene helps this bacterium increase fitness to other cultivars at the population level.

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