Omonrice 12: 147-153 (2004)

SHORT COMMUNICATION

BACTERIAL LEAF BLIGHT RESISTANCE DIAGNOSTICS BY SEQUENCE–TAGGED SITE MARKERS IN RICE (*Oryza sativa* L.)

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ABSTRACT

Bacterial leaf blight caused by the bacterial pathogen Xanthomonas oryzae pv oryzae (Xoo) limits rice yield in all major rice-growing regions of the world, especially in irrigated lowland and rainfed conditions where predisposition factors favor disease development to epidemic proportions. Since bacterial pathogens are difficult to manage, development of host plant resistance is the most effective mean to control disease. One major gene (xa-13) conferring resistance to various races of the pathogen has been identified and utilized in rice breeding programs.

Key work: STS marker, *Xanthomonas oryzae* pv *oryzae*

INTRODUCTION

Bacterial leaf blight (BLB), which is one of the major diseases causes poor development and lowers quality of grain, and increases the number of underdeveloped grains, reduces weight and results in poor maturing and a high proportion of broken rice (Ou 1985). For control it in the field, use of seed from uninfected plants, resistant varieties and careful attention to crop management (for example, by water control, avoidance of damage to seedlings) are most important. Therefore, it is necessary to screen rice varieties (lines) which resist the disease. The advent of molecular markers tagged to different resistance genes enabled convergence breeding and pyramiding of more than two different genes into an agronomic variety. Marker-assisted selection breeding (MAS) has been successfully developed (Huang et al. 1997) for pyramiding four resistance genes into IR24 background.

The present study aims at evaluating of resistant gene polymorphism and assessing phenotypes of resistant lines.

MATERIALS AND METHODS

Materials

(Near isogenic lines [NILs] IRBB5, IRBB53, IRBB0, IRBB4, IRBB13, IRBB21 check) and their recurrent parent IR 24 were used in the study, including 25 lines for selection of BB resistance lines. Local and improved varieties were developed from the selected, special compatible lines to analyze the presence of alleles, which express resistance genes as *xa-5, xa-13* and *Xa-21*. The NILs such as IRBB4 (*Xa-4*), IRBB 5 (*xa-5*), IRBB13 (*xa-13*), and IRBB21 (*Xa-21*) and IR 24 were used for allele designation. The genotypes were used in this study from CLRRI's germplasm

The plants were grown in the field to collect young leaves for DNA extraction.

Methods

DNA isolation

A crude DNA preparation suitable for PCR analysis was prepared using a simplified miniscale procedure (Lang 2002). A single piece of healthy young leaf was harvested and placed in a labeled 1.5 ml centrifuge tube in ice. The leaf sample was macerated using thick glass rod after adding 400 μl of extraction buffer (50 mM Tris-HCl, pH 8.0, 2.5 mM EDTA,300 mM NaCl and 1% SDS). The leaf was grounded until the buffer turned into green colour. After grinding, another 400 μl of extraction buffer was added and mixed by pipetting. The contents were centrifuged at 12,000 rpm in micro centrifuge for 10min. Nearly 400 μl of lysate was extracted with 400 μl of chloroform. The top aqueous supernatant was transferred to another 1.5ml tube and DNA was precipitated with absolute ethanol. The contents were centrifuged for 3 min at full speed and the supernatants were

discarded. The pellet was washed with 70% ethanol. The DNA was air-dried and re-suspended in 50 μl of TE buffer (10mMTris-HCl, pH 8.0, 1 mM EDTA, pH 8.0). One ml of aliquot was used for PCR analysis and the remaining solution was stored at -20^0C for any further use.

Polymerase chain reaction (PCR amplification)

A series of optimization experiments using parents and isogenic lines was carried out in which concentrations of template DNA, primers, dNTPs and *Taq* polymerase were varied to determine, which conditions the PCR reaction mixture of 20 μl contained 25–50 ng template DNA, 50 ng of each primer obtained from CLRRI, 0.05 mM dNTPs, 1X PCR buffer (10 mMTris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.01% gelatin) and 1 unit of *Taq* polymerase obtained from Genome mapping Laboratory, CLRRI. The template DNA was initially denatured at 94^0C for 5 min followed by 35 cycles of PCR amplification under the following parameters: 1 min denaturation at 94^0C, 1 min primer annealing at 55^0C and 1.5–2.0 min primer extension at 72^0C. A final 7 min incubation at 72^0C was allowed for completion of primer extension on thermalcycler. Following amplification, the samples were run on a 1% agarose in 1X TBE buffer stained with 0.1μg/ml of ethidium bromide and visualized under ultraviolet. For PCR-RFLP, initially 5 μl of PCR product was used for gel electrophoresis to determine successful amplification. Seven to eight μl of the PCR product was then used for restriction enzyme digestion if the amplification was successful. The total reaction volume was usually 20 μl. The reaction mixture consisted of 11.4 μl of double distilled water, 2μl of 10 X restriction buffer, 0.6 μl restriction enzyme (10 U/μl) and 6–8 μl of PCR products. The reaction mixture was incubated at 37^0C for 6–8 h. The DNA fragments digested by restriction enzymes were separated on 1.5% agarose gel and visualized under UV light after staining with ethidium bromide.

Bacterial blight screening

The rice plants were inoculated at maximum tillering stage with the Philippine strains of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and one race 11 of CLRRI was used in inoculation. All plants were grown in the greenhouse and inoculated at 55 days after planting. The leaf blades were inoculated by scissors clipping at 5 cm below the leaf tips, the plants were scored as resistant or susceptible reaction by measuring the length of lesion or by visual scoring.

RESULTS AND DISCUSSION

Genetic diversity of plant resistance

A large number of studies conducted at CLRRI have identified the presence of 11 major genes conferring resistance to various races of the pathogen. These genes are designated as *xa-5* and *xa-13*. Differentials of BLB subpopulations were infecting different rice varieties. To characterize the BLB at CLRRI ‘s key screening sites for BLB resistance and to information about the diversity among 67 isolates with 11 races of BLB at Philippine and Vietnam in wet season (WS) in 2003 and 148 local varieties. A previous detailed study of genetic structure within the germplasm collection of local varities was performed based on SSR markers using 100 accessions (Lang et al. 1999). These authors found a low level of SSR variation within accessions with an average percentage of polymorphic loci. Assuming that accessions correspond to populations at equilibrium between genetic drift and mutation in the absence of selection, the expected gene diversity is given by $4N\mu/(1+4N\mu)$ where N is the population size and μ is the mutation rate per locus per generation (Nei 1987). Based on loci, we also found very large variation occurred among races of varieties (figure 1).

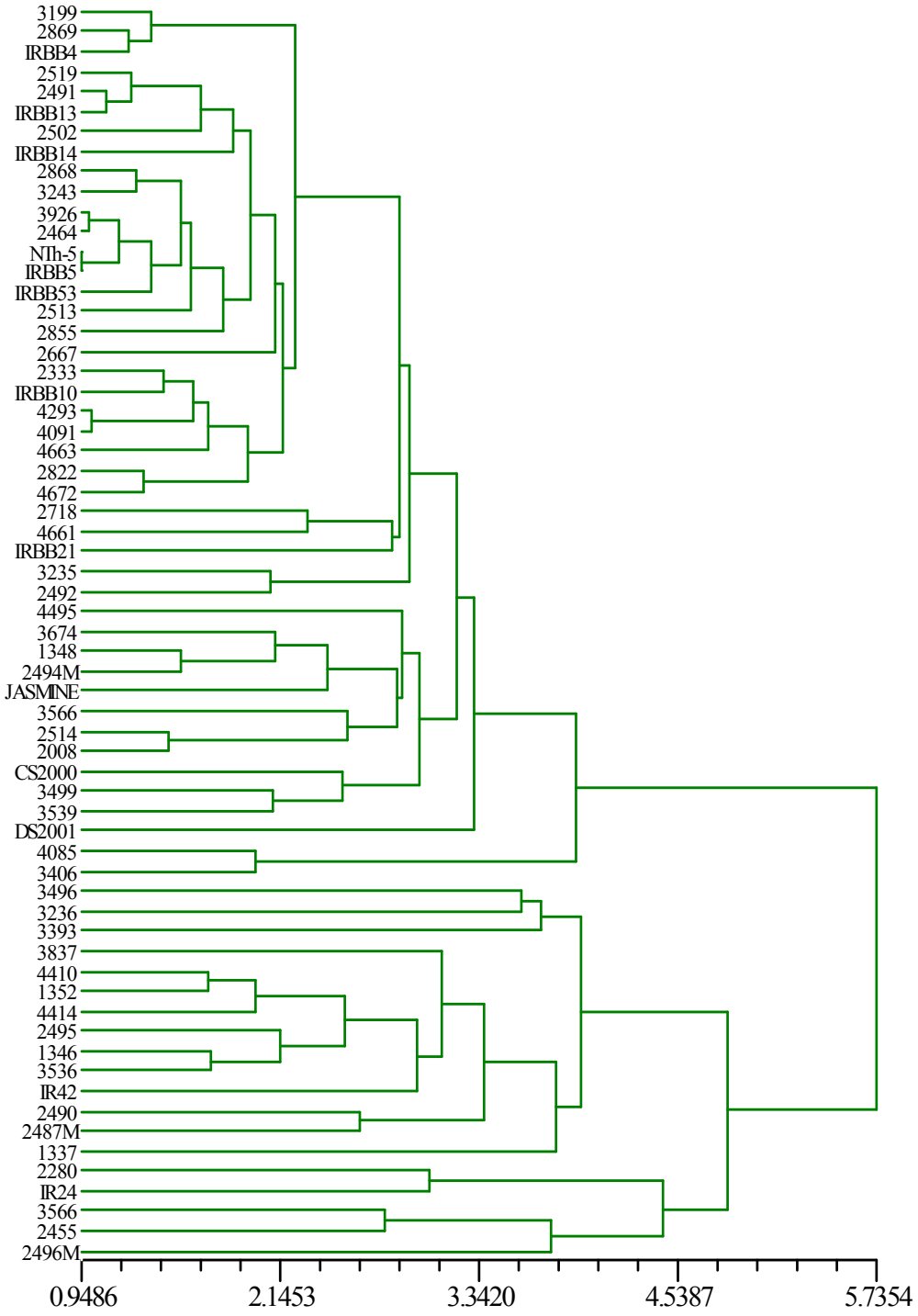

***Figure 1*:** Clustering analysis of 67 cultivars reacted to isolates of BLB at CLRRI , in 2003

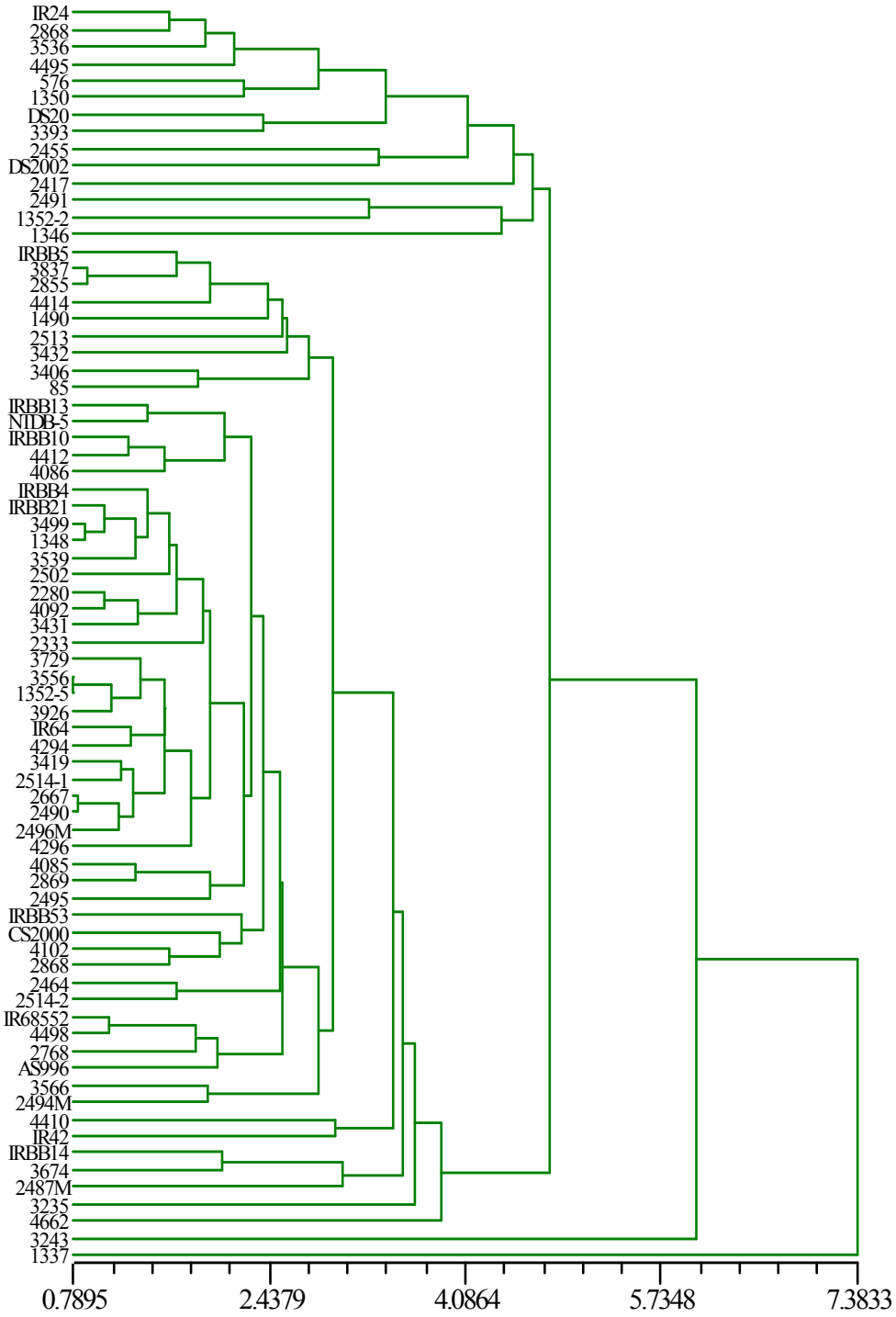

***Figure 2*:** Relationship of rice varieties base on length of the infected leaf in 2004 dry season

Bacterial leaf blight resistance

Leaf inoculation of the 67 varieties and controls produced the expected phenotypes. Almost varieties were scored on a scale 0-5 (table 1 and 2) with the standard of 0-1 as R (resistant), 3-5 as M (moderately resistant), and 7-9 as S (susceptible). The resistant genotype of OM2464 was recorded to infect race PXO 86, 340, 71, 112 and 339. Other eleven resistant genotypes including OM3926 were recorded to race PXO 61, 86, 280, 341.

Table 1: Reaction of nearly isogenic lines in 2003 wet season.

Differential	Bacterial leaf blight race										
	PXO 61	PXO 86	PXO 340	PXO 71	PXO 112	PXO 99	PXO 145	PXO 280	PXO 339	PXO 341	Race11
IR24	S	S	S	S	S	S	S	S	S	S	S
IRBB5	R	M	M	M	R	R	M	M	M	M	M
IRBB13	S	M	M	M	R	M	R	M	R	M	M
IRBB53	M	**R**	M	R	M	M	M	M	M	M	M
IRBB4	M	**R**	M	M	R	M	M	M	M	M	S
IRBB10	**R**	M	M	M	M	M	M	M	M	M	M
IRBB14	M	M	S	M	M	R	R	R	M	M	R
IRBB21	R	R	S	S	S	S	R	M	M	M	M

R: Resistant; M: Moderately resistant; S: Susceptible

Table 2: Reaction of promising varieties in 2003 wet season

Desigantion	Bacterial leaf blight race										
	PXO 61	PXO 86	PXO 340	PXO 71	PXO 112	PXO 99	PXO 145	PXO 280	PXO 339	PXO 341	Race11
OM2491	M	M	M	M	M	M	M	M	R	M	M
OM2502	S	M	M	M	M	M	M	M	M	M	S
OM2868-37	M	M	M	M	M	M	M	M	**R**	M	M
OM3243-10-5	M	M	M	M	M	M	M	M	M	M	M
OM3926-2	**R**	**R**	M	M	M	M	M	**R**	M	**R**	**R**
OM2464-71	M	**R**	**R**	**R**	**R**	M	M	M	**R**	M	M
NTCD-5	M	M	M	**R**	M	**R**	M	M	M	M	M
OM2513	M	**R**	M	M	M	M	**R**	**R**	M	S	M
OM2855-37	M	S	R	M	M	M	M	M	R	M	R
OM4293-4	M	M	M	M	M	M	M	M	M	M	M
OM4091-39	M	M	M	M	M	M	M	M	M	M	M
OM4663	R	M	S	M	M	M	M	M	S	S	M
OM2822	M	**R**	M	M	M	M	M	M	M	**R**	M
OM2718	M	M	M	M	M	S	S	M	M	S	S

R: Resistant; M: Moderately resistant; S: Susceptible

Table 3: Reaction of nearly isogenic lines in 2004 dry season

Differential	Bacterial leaf blight race										
	PXO 61	PXO 86	PXO 340	PXO 71	PXO 112	PXO 99	PXO 145	PXO 280	PXO 339	PXO 341	Race11
IR24	S	S	S	S	S	S	S	S	S	S	S
IRBB5	M	M	M	M	M	M	M	M	R	R	M
IRBB13	M	M	S	M	R	M	M	M	M	M	S
IRBB53	M	R	M	M	R	M	M	M	M	M	S
IRBB4	R	M	M	M	M	M	M	M	M	M	M
IRBB10	M	M	S	M	M	R	R	R	M	M	R
IRBB14	R	R	M	S	M	S	R	M	M	M	M
IRBB21	M	R	M	R	M	M	M	M	M	M	M

R: Resistant; M: Moderately resistant; S: Susceptible

In dry season, resistance genotype with OM1352 was noticed to the infect race PXO340, 71, 112 and 11. Otherwise, OM4086 was recognized to be resistant to race PXO86, 112, 339 and 341. OM3432 was resistant to race 61, 71, 99, 341 and 11. IR64 was resistant to race 61, 71, 99, 280, 339 and 341 (table 5).

Table 4: Reaction of promising rice varieties in 2004 dry season

Varieties	Bacterial leaf blight races										
	PXO 61	PXO 86	PXO 340	PXO 71	PXO 112	PXO 99	PXO 145	PXO 280	PXO 339	PXO 341	Race11
OM1490	M	M	S	M	S	M	S	S	M	S	R
OM3432	R	M	S	R	M	R	M	M	S	R	R
NTDB-5	M	R	S	M	R	R	M	M	M	M	M
OM4086	S	R	M	M	R	M	M	M	R	R	M
OM3499	M	M	M	M	M	M	M	M	M	M	M
OM1348	M	R	M	M	M	M	M	R	R	M	M
OM2280	R	M	S	R	M	M	M	M	M	M	M
OM1352-5	M	M	R	R	R	M	S	M	M	M	R
IR64	R	M	M	R	M	R	M	R	R	R	M
OMCS2000	M	M	M	M	M	M	M	M	S	S	R
AS996	M	R	M	M	R	M	R	M	M	M	S

R: Resistant; M: Moderately resistant; S: Susceptible

Genotype evaluated

The recessive gene *xa-13* confers the resistance to race 6 of *Xoo* originated from the Philippines. The gene was tagged with RAPD marker OPAC05 900 and RFLP marker RG136 and mapped in chromosome 8, using doubled haploid mapping population of IR64 and Azucena (Zhang et al. 1996). The RFLP marker RG136 was used to develop a PCR-based STS marker. The STS primer like *xa-5* STS primer did not produce any polymorphism upon PCR amplification. Hence, the PCR product was digested with *Hin*fI to produce SAP. DNA markers RG136, R2027 in the target region of *xa-13* in chromosome 8 were used to identify BAC clones potentially harboring *xa-13* locus from a rice BAC library (Sanchez et al. 1999). The study resulted in development of a PCR-based marker RG136 that is more powerful for MAS involving closely related parents because it shows polymorphism among Nep Da, Nep Thơm, Nep Lun, Vephich, Gay Xe, Ban 157, Nep Cai Hai Duong, which expressed *xa-13*. Almost improved varieties in the experiment were susceptible, it means no expression of *xa-13*

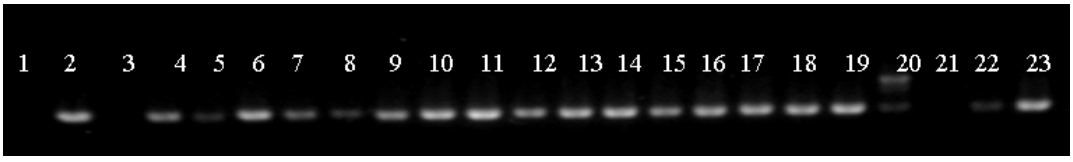

Figure 3: PCR products at locus RG136
Lane 1 – 23: OM2280, OM2717-150-3, OM3236-8, OM3926-2, OM3536, OM2718-2, OM2718-3, OM3240-80, OM2718-1, OM3234-10, IR65610, OM3235-105, OM2822-1, OM2500, OM2008, OM2008-1, IRBB13, IR68552, Acc285, Acc293, Acc296, Acc297, Acc299.

To develop a PCR-based STS marker for use in marker-assisted selection, the STS marker was found to give monomorphic banding pattern on PCR amplification between the resistant and susceptible plants. Hence, the PCR product was digested with a restriction enzyme *Dra*I to generate specific amplicon polymorphism (SAP). Sanchez et al. (2000) have successfully cloned the *xa-5* region in chromosome 5. They identified two bacterial

artificial chromosome (BAC) clones 9E8 and 28N22, which contained *xa-5* locus. The cDNA clone 5P2 was identified by both the BAC clones following colony hybridization. Both the cDNA clone and the BAC clones were used for transformation experiments and sequencing. The recessive resistance gene *xa-5* was tested with markers RG556, polymorphism was not recorded among 148 local varieties and improved ones.

CONCLUSION

In 2003 wet season and 2004 dry season, some promising rice varieties were clearly distinguished from resistant to susceptible clusters.

There are OM3536, DS20 and OM4495 varieties in susceptible cluster and OM3432, OM1352-5, OM2718, OM2822 and OM3432 in resistant one.

At locus RG136, both resistant gene-linked bands and susceptible gene-linked bands were presented in Nepda (Acc293), Nep Thơm, Nep Lun, Vephich, Gay Xe, Ban 157, Nep Cai Hai Duong

At locus RG556, polymorphism was also recorded, but no local varieties and improve varieties in the experiment were noticed to have the resistant gene.

Marker-assisted selection can be successfully used to detect *xa-13* gene. Molecular markers available for various resistance genes can be used to combine these genes into one of the parents in the most population in breeding program.

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SUMMARY IN VIETNAMESE

Chẩn đóan tính kháng bệnh bạc lá lúa bằng STS marker

Bệnh bạc lá lúa do vi khuẩn *Xanthomonas oryzae* pv *oryzae* (*Xoo*) gây ra, với hậu qủa làm thất thóat năng suất,nhất là trong điều kiện canh tác lúa có nước tưới là lúa nước trời. Bệnh bạc lá lúa là đối tượng rất khó kiểm sóat bằng thuốc do đó, giống kháng là giải pháp được nhiều nhà chọn giống quan tâm. Gen kháng *xa-13* có khả năng kiểm sóat được nhiều nòi gây độc tính cao ở miền Nam đã được nghiên cứu tại locus RG136, định vị trên nhiễm sắc thể số 8.

Các giống lúa OM3432, OM1352-5, OM2718, OM2822 và OM3432 được ghi nhận có chứa gen kháng *xa-13*, tương tự như các giống lúa địa phương cho gen kháng này: Nếp dà (Acc293), Nếp Thơm, Nếp Lùn, Vệ phích, Gảy Xe, Ban 157, Nếp Cái Hải Dương.
