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Original Research Article

Blast Resistant Genes Distribution and Resistance Reaction to Blast in Korean Landraces of Rice (*Oryza sativa* L.)

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Abstract - Rice blast (*Magnaporthe oryza* B.) is one of the most important diseases in rice that causing great yield losses every year around the world. It is important to screen valuable genetic resources for improving blast resistance. This study was conducted to identify the blast resistance in 279 Korean rice landraces using blast nursery tests and isolate inoculum screening. The results showed that 11 landrace accessions found to be resistant to rice blast in blast nursery and inoculation screening tests and the degree of lesions in most accessions showed that they were susceptible to reactions. In order to find the distribution of blast resistant genes, a molecular survey was conducted to identify the presence of major blast resistance (R) gene in 279 Korean landraces. The results revealed that their frequency distribution was *Pik-m* (36.2%), *Piz* (25.4%), *Pit* (13.6%), and *Pik* (10%). Besides, the frequency distribution of *Piz-t*, *Pii*, *Pik-m/Pik-p*, *Pi-39(t)*, *Pib*, *Pi-d(t)2*, *Pita/Pita-2* and *Pi-ta* genes were identified as less than 10%. The results did not consist with the reactions against blast diseases between genotypes and phenotypic part of the nursery tests and isolate inoculation. For concluding these results, we used genome-wide SSR markers that have closely been located with resistance genes. The PCoA analysis showed that the landrace accessions formed largely two distinct groups according to their degree of blast resistance. By comparing genetic diversities using polymorphic information contents (PIC) value among the resistant, total and susceptible landraces, we found that PIC values decreased in four SSR markers and increased in six markers in the resistant accessions, which showed contrary to total and susceptible groups. These regions might be linked to resistance alleles. In this study, we evaluated the degree of blast resistance and the information about the distribution of rice blast resistant genes in Korean rice landraces. This study might be the basis for association analysis of blast resistance in rice.

Key words - Rice landraces, Blast, *M. grisea*, Molecular marker

Introduction

Rice (*Oryza sativa* L.) is one of the important staple food crops in the world and maintaining stable rice production is extremely important to feed the constantly growing human population (Maclean *et al*., 2002; Sasaki and Burr, 2000). Manipulation of the disease resistant in rice is an important objective in all rice-breeding programs, because the production is constantly affected by several major diseases, such as bacterial blight, blast, sheath blight, and tungro. Among these diseases, rice blast disease caused by the ascomycete fungus *Magnaporthe oryzae* (Couch and Kohn, 2002), as the leading cause of yield loss of rice worldwide is the most devastating fungal disease on cultivated rice as well as other species of the Poaceae (Zeigler *et al*., 1994; Talbot and Foster, 2001; Talbot, 2003). For the control of blast diseases, the breeding of resistant varieties is an effective approach to reduce the use of pesticides and minimized rice losses due to this disease. However, the breakdown of blast resistance frequently occurs due to the rapid change in the blast pathogenic races after new developed cultivar release, with the exception of the some elite cultivar. It is indicated that the genetic control of blast resistance is complex due to major and minor genes with complementary or additive effects, as well as their environment interactions (Yamanaka and Yamaguchi, 1987; Yaegashi, 1994; Han *et al*., 2001). The ideal approach for blast control is probably to develop durable resistance cultivars accumulating several resistance (R) genes against highly variable pathogen

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(Bonman *et al*., 1986; Hittalmani *et al*., 2000).

Genetic studies of resistance to rice blast began by establishing the differential system for races of the blast in the early 1960s. Since then, the inheritance of resistance has been extensively studied, and over 70 major R genes have now been reported (Chen *et al*., 2005; Ballini *et al*., 2008; Koide *et al*., 2009). During the last few years, considerable progress has been made to understand molecular mechanisms of *M. grisea* infection to rice plants and rice blast R genes [*Pit, Pib, Piz, Pik, Pik-m/Pik-p, Pita/Pita-2* (Hayashi *et al*., 2006), *Piz-t* (Hayashi *et al*., 2004; Zhou *et al*., 2006), *Pi-d(t)2* (Chen *et al*., 2004), *Pii* (Jeon *et al*., 2003), *Pik-m* (Zhai *et al*., 2011), *Pi39* (Liu *et al*., 2007), *Pita* (Jia *et al*., 2002 and Jia *et al*., 2004), *Pi9* (Qu *et al*., 2006), *Pid2* (Chen *et al*., 2006), *Pi2* (Zhou *et al*., 2006), *Pi36* (Liu *et al*., 2007), *Pi37* (Lin *et al*., 2007), *Pi5* (Lee *et al*., 2009) and *Pi3* (Shang *et al*., 2009)] have been identified and characterized via map-based cloning or chromosome walking. Due to map-based cloning marker system and the integration of high-density molecular linkage map with genome sequence information of rice, marker-assisted selection (MAS) in molecular marker technology may provide new solutions for identifying and pyramiding valuable gene to improve the blast disease resistance in rice. Some studies have been reported that introductions of the major resistant genes into the rice varieties are correlated to the blast disease resistance of rice (Cho *et al*., 1996; Jeung *et al*., 2007; Suh *et al*., 2009) using molecular marker that reported blast resistant (R) gene marker or known as well-characterized resistant gene markers. According to the widely genome research progresses, specific gene marker identified in accordance with the characteristics serve as a valuable tool for assessment of genetic variability and information offering for high blast resistance among rice landraces germplasm.

According to Harlan (1975), landraces are the balanced resources in equilibrium with both the environment and pathogens and are genetically dynamic. Rice landraces have been domesticated for a long time in a specific region and accordingly were adapted to their natural and cultural environment. The landraces has a long term co-evolution between the resources and rice blast pathogen, and the distribution of resistance genes and rice blast was closely related (Li *et al*., 2012). Landrace could be used for breeding program in that it conserves useful alleles adapted to specific environment. Variation in landraces can be used to complement and is helpful for broadening the crop gene pool (Kobayashi *et al*., 2006). For these reasons, rice landraces has been valued as genetic variation sources of many agronomic traits and may have great potential for breeding of cultivated rice (Villa *et al*., 2006). Korean landraces have been continuously maintained by farmers within different agricultural areas as well as their local environments. Therefore, this study was conducted to get the blast resistance degree of rice landraces germplasm in blast pathogenicity of field and *in vitro* tests and to investigate the distribution of blast resistant genes in Korean landraces. The present study reports on the screening and evaluation of rice germplasm for sources of resistance against rice blast disease.

Materials and Methods

Evaluation of blast resistance by field nursery test and *in vitro* inoculation

The plant materials used in this study were 279 Korean rice landraces (Table 1), which have been conserved in the RDA Genbank (http:// genebank.rda.go.kr.). These rice germplasm were evaluated for seedling reaction under field nursery test, which was replicated two times at experimental field of Cheolwon, in Korea from 2010 to 2011. Each accession was planted in one row of 50 ㎝ long and 15 ㎝ apart, with cv. mixture of three cultivars (Chugwangbyeo, Odaebyeo, and Yeomyeongbyeo) as a susceptible check from each side of the bed and alternatively after the ten rows of each tested entry. Plants were left for natural leaf blast infection and scored 30-45 days after sowing.

M. grisea isolates collected in Korea was used for pathogenic test in this study. The 31 isolates of *M. grisea* have been conserved in the National Institute of Crop Science (NICS) and these isolates are genetically stable and routinely used for studies in Korea (Ahn *et al*., 2000; Goh *et al*., 2013). Pathogen inoculation and blast response evaluation of the 279 landraces were carried out at the NICS, Rural Development Administration, in Korea. At the seedling stage, the inoculums suspension of ca. 5×10^4 conidia/㎖ was sprayed onto rice leaves in a controlled chamber. Inoculated seedlings were moved to the greenhouse after 3 days incubation in saturated humidity

Table 1. List of Korean rice landraces used in this study

Stock No. of Genebank	Accession name	Stock No. of Genebank	Accession name	Stock No. of Genebank	Accession name	Stock No. of Genebank	Accession name
IT004688	Ggaebyeo	IT005693	Dadujo	IT006266	Banchonjo	IT007446	Agukdo
IT004692	Gasanbyeo	IT005694	Damagung	IT006298	Baekkiongzo	IT007458	Arongbyeo
IT004694	Gaksijeomjo	IT005716	Dabaekjo	IT006302	Baekgogna	IT007460	Anna
IT004753	Gangdodo	IT005718	Daigolbyeo	IT006310	Baekgwangok	IT007559	Aengmi
IT004760	Gangreungdo	IT005736	Daigolna	IT006328	Baekmangjo	IT007585	Yeobyeo
IT004768	Gangsanbyeo	IT005742	Daegoldo-1	IT006366	Baekjanggun	IT007592	Yeosubyeo
IT004769	Gangbaedo	IT005743	Daegoldo-2	IT006372	Baekjo	IT007596	Yeoussalbyeo
IT004770	Gangwondo	IT005754	Daegwando	IT006376	Baekjicheongbyeo	IT007598	Yeonanjo
IT004771	Gangwonna	IT005756	Daeguna	IT006380	Baekchalbyeo	IT007605	Yeolsulbyeo
IT004775	Gangcheongdo	IT005762	Daegudo	IT006385	Baecheon-1	IT007622	Yejo
IT004811	Ge	IT005835	Daejodo	IT006386	Baecheon-2	IT007629	Orido-1
IT004839	Gyeongjobaekjo	IT005882	Danduna	IT006397	Baekhyangjo	IT007630	Orido-2
IT004899	Gwaksanjo	IT005893	Dangdo	IT006400	Beodeulbyeo	IT007631	Orido-3
IT004914	Gwansansaek	IT005908	Dorae	IT006410	Beobpanhwa	IT007633	Obaekjo
IT005046	Guwoldo	IT005915	Doaji	IT006520	Buldo	IT007684	Olmutge
IT005051	Gujungdo-1	IT005946	Dongsanjo-1	IT006522	Buljo	IT007688	Olbyeo
IT005052	Gujungdo-2	IT005948	Dongsanjo-2	IT006551	Sandadagido	IT007693	Olwaedu
IT005057	Gucheondo	IT005970	Dongobyeo	IT006554	Sando-1	IT007742	Yongmyeonheuk
IT005068	Guhwangdo-1	IT005980	Dudo	IT006556	Sando-2	IT007746	Yongcheon-1
IT005076	Gunjo	IT005989	Duchungjong	IT006559	Sandudo-1	IT007747	Yongcheon-2
IT005095	Gwido	IT005994	Deokjeokjodo	IT006560	Sandudo-2	IT007792	Wonjabyeo
IT005126	Geumdo	IT006000	Deulleongdeulchigi	IT006578	Ssalbyeo	IT007807	Yu
IT005133	Geumjeomdo	IT006010	Ddangjo	IT006596	Samgyeongjo	IT007900	Yukwoljo
IT005142	Geumchangdo	IT006066	Maekjo	IT006620	Sangdo-1	IT007903	Eumeuchal
IT005205	Na-1	IT006078	Monajo	IT006622	Sangdo-2	IT007975	Eunjo
IT005216	Naengdo	IT006084	Modo-1	IT006663	Seorianjeunbaengi	IT007981	Eumjo
IT005504	Noinjo-1	IT006087	Modo-2	IT006684	Seoksanna	IT007999	Irakdo
IT005505	Noinjo-2	IT006089	Mojo	IT006687	Seoksanjo	IT008189	Icheonchunggu
IT005506	Noindo-1	IT006100	Monggeunchanarak	IT006699	Seondal	IT008199	Inbujinado
IT005508	Noindo-2	IT006103	Mudaraegi	IT006735	Sodujo	IT008267	Jangsamdo-1
IT005509	Noindo-3	IT006114	Musaek Jojeokjo	IT006768	Soemeoribyeo	IT008268	Jangsamdo-2
IT005657	Nokdudo-1	IT006116	Muando	IT006772	Soemeorijijang	IT008277	Jangjo-1
IT005660	Nokdudo-2	IT006119	Muyeopseoldo	IT006776	Soebenchigi	IT008278	Jangjo-2
IT005677	Neuseubyeo	IT006125	Mujudo	IT007245	Suwonjo	IT008286	Jaeraesuyeom
IT005679	Dadajo-1	IT006129	Migwang	IT007254	Sujungjo	IT008289	Jaeraedo
IT005681	Dadajo-2	IT006138	Mido	IT007268	Ssubyeo	IT008293	Jaeraejodo-1
IT005682	Dadajo-3	IT006151	Mijo	IT007270	Sukna-1	IT008295	Jaeraejodo-2
IT005683	Dadajo-4	IT006243	Badolbyeo	IT007274	Sukna-2	IT008314	Jeosaekdo
IT005689	Dadeogbereum	IT006247	Baramdungguri	IT007278	Sulsuldo	IT008355	Jeokmosaek
IT005691	Dadoaek	IT006258	Bandalbyeo	IT007442	Agudo	IT008357	Jeokbakna

Table 1. Continued

Stock No. of Genebank	Accession name	Stock No. of Genebank	Accession name	Stock No. of Genebank	Accession name	Stock No. of Genebank	Accession name
IT008361	Jeoksudangan	IT008983	Palcheondo-2	IT009233	Heukdaegu	IT010707	Pocheonryukdo
IT008382	Jeonggeumjo	IT008986	Pyeongbuk 4	IT009243	Heuksaekdo	IT010721	Hwaseongbatchal
IT008385	Jeonggijosaeng	IT008992	Pyeongyang	IT009244	Heukjeodo-1	IT010726	Heukmok
IT008390	Jeongrakdo	IT008999	Pyodo	IT009245	Heukjeodo-2	IT010727	Heukpi
IT008401	Jeongjo	IT009056	Hangibuji	IT009250	Heukjeongdo	IT110944	Sandu Chalbyeo
IT008408	Jeongjonghwa	IT009057	Hannaebyeo	IT009251	Heukdo	IT151696	Weonsanchalbyeo
IT008453	Jodo	IT009059	Hansujindo	IT009265	Heuindadak	IT155895	Daejichal
IT008471	Jodujo	IT009060	Hanyangjo	IT009267	Heuinbe	IT155896	Joslbichal
IT008528	Joseokdo	IT009065	Hambureubyeo 3	IT009590	Hongcheonchal	IT155897	Guisimchal
IT008530	Joseondo	IT009069	Haerim	IT009797	Sundal	IT173444	Jagwangdo-1
IT008579	Jojeongdo	IT009073	Haejo	IT010151	Gawichal	IT173445	Jagwangdo-2
IT008580	Jotajo	IT009077	Haengpung	IT010161	Gangweondo	IT173446	Ginggalagsyale
IT008590	Jongjobaekjo	IT009117	Hongna	IT010275	Neulbyeo	IT203619	Jagwangdo-3
IT008599	Jujodo	IT009118	Hongdo	IT010339	Batnarak-1	K026144	Daegujo
IT008672	Junganjeunbaengi	IT009120	Hongdodo-1	IT010340	Batnarak-2	K026145	Sirori
IT008710	Jindo	IT009123	Hongdodo-2	IT010345	Beongok	K026146	Baekgokna
IT008732	Chanarak-1	IT009129	Hongsaekdo	IT010374	Sando-1	K026147	Okcheong
IT008734	Chanarak-2	IT009138	Hongcheongdo	IT010375	Sando-2	K026148	Donna
IT008743	Chanseobyeo	IT009142	Hwado	IT010376	Sandudo	K026149	Yeonnado
IT008749	Chalbyeo	IT009169	Hwangdaiolbyeo	IT010480	Yongdalichalbyeo	K026150	Mumojojeokjo
IT008799	Cheonjudo	IT009172	Hwangjo-1	IT010555	Yukseongjaerae	K026153	Seon
IT008806	Cheonpungdo	IT009173	Hwangjo-2	IT010565	Icheon 7 ilchal	K026155	Chalbyeo
IT008831	Cheongsongjo	IT009174	Hwangjo-3	IT010577	Jangmangjaerae	K026158	Seungna
IT008883	Chigyeongdo	IT009177	Hwangjo-4	IT010582	Jaeraeyukdo	K026159	Akkudichal
IT008888	Chimabyeo	IT009180	Hwangjodo	IT010612	Jodo	K026160	Daeguna
IT008895	Chindadachigi-1	IT009182	Hwangju	IT010625	Josaengjodo	K026162	Yongjo
IT008897	Chindadachigi-2	IT009189	Hwangtodo	IT010627	Joseokjo	K026165	Chullaesan
IT008951	Paldado	IT009191	Hwangtojo	IT010630	Jjok-Je-Bi-Chal-1	K026151	Nado
IT008981	Paljungsu	IT009192	Hwanghaedo	IT010631	Jjok-Je-Bi-Chal-2	K026194	Monggeunchal
IT008982	Palcheondo-1	IT009221	Hyoseongjaeraejong	IT010704	Pocheonyumangchal		

chamber. Blast disease incidence was evaluated in two weeks after inoculation according to Ahn *et al*. (2000). The incidence of blast disease in blast nursery test was scored from 0 (no lesions) to 9 (necrosis of all leaves and sheaths) following Standard Evaluation System (SES, IRRI, 2002). Accessions were assigned to the resistance (R) group in score 0-3, moderate resistance in 4-6 and susceptible in 7-9, respectively. The reproducible accessions of R and S group were selected to analyze the blast resistance gene distribution. The isolate inoculation screening were assigned to resistance in score 0-2, moderate resistance in 3, and susceptible in 4-5, respectively.

DNA extraction and Marker analysis

Genomic DNA as extracted from two weeks old fresh leaf tissue according to modified CTAB method as previously described by Kump and Javornik (1996). The DNA concentration was determined using a UV−Vis spectrophotometer

Table 2. Gene specific primers used for the amplification of rice blast resistance genes

Linked gene	chromosome	Marker	Primer sequence Forward(5′-3′)	Type of marker	size range	Reference
Pit	1	t311	F-CGTGAACCCAAGGCACCAGTATTA F-CGTGAACCCAAGGCACCAGTATTC R-CATGTAGTTCTGGATGTTGTAGCTACTC	SNP	287	Hayashi *et al*., 2006
Pib	2	b213	F-GCATTAGATAGTGATGAAAGCCGG F-GCATTAGATAGTGATGAAAGCCGA R-TGTTCATCCAGGCAATTGGC	SNP	218	Hayashi *et al*., 2006
Piz-t	6	zt3545	F-CAAGCCTAGCGAGCTCGAGCGCC F-GGGCTATGAAAAAAGCTCAGAGATC R-AAGCCTCTGCAGCTTCTCTGGTG	InDel		Hayashi *et al*., 2004
Piz	6	z56592	F-GGACCCGCGTTTTCCACGTGTAA F-GGACCCGCGTTTTCCACGTGTAC R-AGGAATCTATTGCTAAGCATGAC	InDel	292	Hayashi *et al*., 2006
pi-d(t)2	6	RM3	F-ACACTGTAGCGGCCACTG R-CCTCCACTGCTCCACATCTT	SSR	145	Chen *et al*., 2004
Pi5	9	JJ113-T3	F- GGATGATGTGATCTGCAGAG R- CTCTTGGTGATCTTTGTTAC	STS	484	Jeon *et al*. 2003
Pik	11	k6438	F-GCGACCCTGTCTTTGGACTGG F-GCGACCCTGTCTTTGGACTGC R-GAATGATGAGGAGAGAAGGCTGTCG	SNP	226	Hayashi *et al*., 2006
Pik-m	11	dCAPS-685	F-TCGCCGGTGACCTAAGAGAT R-GATTTCACCGGCGCAAGCAT	SNP	180	Zhai *et al*. 2011
Pik-m/Pik-p	12	k641	F-GCTGGGACACCAACATCCATGC F-GGCTGGAACACCAACATCCATGG R-GCGCTGGACTTGGAACTAGTGC	SNP	387	Hayashi *et al*., 2006
Pita, Pita-2	12	ta5	F-CAGCGAACTCCTTCGCATACGCA F-CAGCGAACTCCTTCGCATACGCG R-CGAAAGGTGTATGCACTATAGTATCC	InDel	515	Hayashi *et al*., 2006
Pita	12	YL155/87 YL183/87	F-AGCAGGTTATAAGCTAGGCC F-AGCAGGTTATAAGCTAGCTAT R-CTACCAACAAGTTCATCAAA		1042	Jia *et al*. 2002, 2004
pi39	12	39M12	F: GGAAACTCCAGGTGTGATAGG R: AACGATGCTCTGGTGCTCTC	STS	259	Liu *et al*., 2007

(ND-1000; NanoDrop, Wilmington, DE, USA). The DNA solution was then diluted to a working concentration with distilled water and stored at -20℃ until use. Polymerase chain reaction (PCR) was performed using the gene specific primers described as blast resistant and susceptible genes, which are listed in Table 2. Ten ng of genomic DNA was used in a 20 μl PCR reaction containing 2 μl of the specific primer pairs (10 pmol/ul), 2.0 μl of 10 x PCR buffer, 1.6 μl of dNTP (2.5 mM), and 0.2 μl of *Taq* polymerase (5 unit/μl; Promega. USA). The reaction mixture was subjected to the following conditions: initial denaturation at 94℃ for 3 min, followed by 35 cycles of denaturation at 94℃ for 30 s, annealing at 50-55℃ for 45 s and extension at 72℃ for 45 s and final extension at 72℃ for 10 min. PCR was carried out in PTC-220

Table 3. Distribution patterns of blast resistant genes in 11 resistant cultivars selected on blast nursery test and isolate inoculation

IT/Tem.	Accession Name	*Pit*		*Pib*		*Piz-t*		*Piz*		*Pik*		*Pik-m*	*Pik-m, Pik-p*		*Pita, Pita-2*		*Pi-ta*		*Pi39*		*Pi-d(t)2*	*Pii*
		t311		b213		zt3545		z56592		K6438		dCAPS-685	K641	ta5	YL 155	YL 183	39M12		39M22		RM3	JJ113-T3
		R[z]	S[y]	R	S	R	S	R	S	R	S	R	R	S	R	S	R	S	R	R	R	R
IT006538	Saducho	+	-	+	-	-	+	-	+	-	+	+	+	-	-	+	-	+	+	+	-	-
IT009118	Hongdo	+	-	+	-	-	+	-	+	-	+	+	-	+	-	+	-	+	+	+	-	-
IT009221	Hyosung-jaerae	+	-	+	-	-	+	-	+	-	+	-	-	+	+	-	+	-	+	+	+	-
IT010345	Beonkok	+	-	-	+	-	+	-	+	-	+	-	-	+	-	+	-	+	-	-	-	-
IT010480	Yongdali-chalbyeo	+	-	-	+	-	+	-	+	-	+	-	-	+	-	+	-	+	-	-	-	-
IT010555	Yukseong-jaerae	+	-	-	+	-	+	-	+	-	+	-	-	+	-	+	-	+	-	-	-	-
IT010565	Icheon7ilcha	+	-	-	+	+	-	-	+	-	+	-	-	+	-	+	-	+	-	-	-	-
IT010577	Jangmang-jaerae	+	-	-	+	-	+	-	+	-	+	-	-	+	-	+	-	+	-	-	-	-
IT010721	Hwaseong-batchal	+	-	-	+	-	+	-	+	-	+	-	-	+	-	+	-	+	-	-	-	-
IT010726	Hweukmok	+	-	-	+	-	+	-	+	-	+	-	-	+	-	+	-	+	-	-	-	-
K026194	Mongkeun-chalbyeo	+	-	-	+	+	+	-	+	-	+	-	+	-	-	-	-	-	+	+	-	-

[+]: Positive response, -: negative response
[z]blast resistance specific primers
[y]blast susceptible specific primers

Table 4. Chromosomal localization, number of detected alleles and polymorphism information content (PIC) obtained through 64 SSR marker analyses

SSR Marker	Chr. No[z]	N_A[y]	PIC[x]	SSR Marker	Chr. No.	No. of alleles (Na)	PIC
RM1	1	14	0.570	RM197	6	6	0.083
RM5	1	6	0.617	RM527	6	8	0.408
RM580	1	13	0.844	RM133	6	4	0.144
RM246	1	9	0.709	WxOligo	6	9	0.497
RM259	1	10	0.809	RM418	7	15	0.769
RM237	1	5	0.452	RM1306	7	24	0.843
RM174	2	5	0.159	RM3718	7	8	0.457
RM48	2	27	0.811	RM11	7	8	0.312
RM3857	2	15	0.843	RM118	7	3	0.021
RM6165	2	3	0.072	RM149	8	10	0.605
RM12676	2	3	0.400	RM44	8	12	0.741
RM208	2	4	0.347	RM310	8	18	0.828
RM154	2	11	0.418	RM23455	8	4	0.433
RM135	3	6	0.099	RM210	8	11	0.734
RM3766	3	12	0.763	RM408	8	5	0.109
RM231	3	8	0.496	RM433	8	8	0.461
RM232	3	19	0.877	RM444	9	10	0.428
RM514	3	7	0.381	RM257	9	11	0.647
RM252	4	8	0.306	RM3533	9	6	0.268

Table 4. Continued

SSR Marker	Chr. No[z]	N_A[y]	PIC[x]	SSR Marker	Chr. No.	No. of alleles (Na)	PIC
RM349	4	11	0.533	RM5515	9	6	0.541
RM241	4	14	0.792	RM215	9	6	0.590
RM6629	4	5	0.216	RM171	10	5	0.121
RM16427	4	4	0.236	RM228	10	12	0.671
RM307	4	6	0.096	RM6144	10	3	0.264
RM13	5	8	0.270	RM271	10	4	0.042
RM249	5	21	0.904	RM206	11	41	0.933
RM3322	5	7	0.293	RM21	11	11	0.577
RM19159	5	14	0.650	RM519	12	5	0.049
RM31	5	12	0.529	RM247	12	13	0.693
RM413	5	11	0.496	RM277	12	2	0.035
RM3616	5	6	0.524	Mean		9.6	0.472
RM103	6	6	0.238	Min.		2	0.021
RM253	6	11	0.668	Max.		41	0.933
OSR21	6	6	0.505				

[z]Chromosome Number.
[y]Number of alleles.
[x]Polymorphic information content.

thermocyclers (MJ Research, Waltham, MA, USA). The PCR products were then run on a QIAxcel capillary gel electrophoresis system according to the manufacturer's instructions (Qiagen, Germany) and fragments were sized and scored using QIAxcel ScreenGel software (Qiagen, Germany).

Assess of microsatellite markers

The M13-tail at the 5'-end region PCR method was used to measure the sizes of the amplified products of SSRs as previously described by Schuelke, (2000). For genotyping analysis, primers were chosen from the Gramene database (http://www,gramene.org/markers/microsat/ssr.html) and the genome-wide SSR markers used in this study are listed in Table 4. Amplified fluorescent-labeled PCR products were analyzed in an ABI-Prism 3130x1 Genetic Analyzer (Applied Biosystems). Fragments were sized and scored into alleles using GeneMapper v4.0 (Applied Biosystems), and the individual fragments were assigned as alleles.

The software PowerMarker version 3.25 (Liu and Muse, 2005) was used to calculate the number of alleles (N_A) and polymorphic information content (PIC), and constructed based on a genetic distance matrix from SSR genotyping results. We conducted principal coordinate analysis (PCoA) of individual genotypes using the software, GenAlEx version 6 (Peakall and Smouse, 2006), to complement the output of the phylogenetic analysis.

Results

Evaluation of blast resistance in blast nursery test and *in vitro* inoculation

To get the blast resistance degree in rice landrace germplasm, a total of 279 Korean landraces were used and then applied in blast nursery test and *in vitro* inoculation. The responses to *M. grisea* were indicated divergent reactions from 0 to 9 (a scale 0 = no lesions to 9 = dead leaves) based on the Standard Evaluation System (SES) for rice in IRRI (IRRI, 2002). The infection type in rice genotypes showed that genotypes have been in largely three groups, which are resistant (infection type 0 to 1), moderate (infection type 4 to 5) and susceptible (infection type 8 to 9). Among these accessions, 3.9% genotypes are located in the resistant group, 73.5% genotypes are located in susceptible group. The lesions degree and the number of resistant and susceptible accessions equated to results of blast response in 2010 and 2011 (Fig. 1). The incidence degree of most accessions showed the diverse

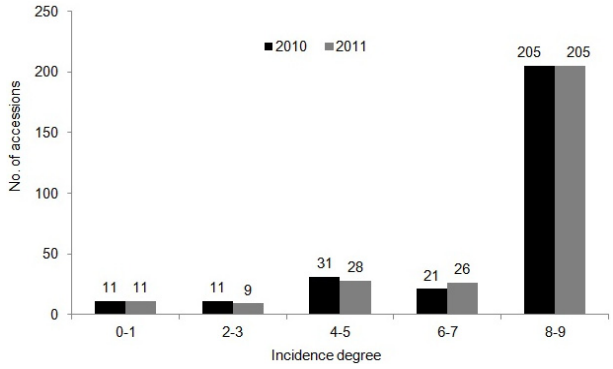

Fig. 1. Frequency distribution of disease incidence degree at blast nursery test of 279 landrace at field in 2010 and 2011.

reactions in both the blast nursery test and *in vitro* inoculation. Eleven accessions were found to be resistant 0 (no lesion absorbed) or 1 (small brown specks of pin point size) against rice blast at experimental field. Two hundred five highly susceptible accessions were scored as 8 or 9 that showed over 3 ㎝ of blast lesion or many leaves were dead by longer infection of over than 50% died leaf area. Although some of moderately resistant accessions were changed to susceptible or showed highly range of variation, the 11 accessions (IT006538, IT009118, IT009221, IT010345, IT010480, IT010555, IT010565, IT010577, IT010721, IT010726, and K026194) were revealed constantly resistance in both years (Fig. 1). Thus, these 11 accessions were tested for accurately screening at the seedling stage against 31 isolates mixture of *M. grisea* according to Goh *et al*. (2013).

To screen blast resistant accessions at the seedling stage, Greenhouse screening of rice landrace accessions were conducted *in vitro* inoculation to leaf blast. In all, 20 accessions resistant (≤ 2.0 score on a 0-to-5 scale), 54 accessions were moderately resistant (3.0 score), and 205 accessions were susceptible (4.0 to 5.0 score) to blast isolates (Data not shown). Of the 20 leaf blast-resistant accessions, 11 and 9 landrace accessions were also found resistance and moderately resistance in the blast nursery screens during both years, respectively. The selected accessions in our study will be used to increase the blast resistance in the rice breeding program for the development of disease resistant commercial cultivars after determining confirmation their genetics, if these are found to possess other desirable agronomic characters.

Estimation of genotypes for blast resistance genes

We have conducted to get the blast resistance degree of Korean landraces of rice to blast disease on field tests and *in vitro* inoculation. However, because the bioassay to rice blast in the field shows high variations influenced by the environment, it is important to screen valuable genetic resources through molecular approaches for improving blast resistance. This part has performed to acquire information for the amplification patterns of resistance genes against rice blast disease in the 279 landrace accessions using 12 major blast resistant genes such as *Pit, Pib*, *Piz-t*, *Piz, Pi-d(t)2, Pii, Pik, Pik-m, Pik-m/Pik-p, Pi-ta/Pita-2, Pita and Pi39* for improving rice landrace breeding efficiency. These resistant (R) genes found that *Pik-m* and *Piz* genes amplified in the 101 (36.2%) and 71 (25.4%) accessions, respectively. *Pit*, *Pik*, *Piz-t, Pii* and *Pik-m/Pik-p* genes were observed ranged from 11 (3.9%) - 38 (13.6%) in tested accessions, but *Pi-39(t)*, *Pib, Pid(t)2*, *Pita/Pita-2*, *Pita* genes were identified in less than 10 accessions (Fig. 2A). The *Pik*-multiple allele genes, *Pik*, *Pik-m*, and *Pik-m/Pik-p*, differently identified in 279 Korea landraces in this study. The *Pik*-m of these genes amplified in 101 accessions, whereas the *Pik* and *Pik-m/Pik-p* genes showed in 28 and 11 accessions, respectively. The rice blast resistance gene *Pik-m*, which is one of the alleles located at the well-known *Pik* locus on chromosome 11, confers high and stable resistance to many Chinese rice blast isolates (Zhai *et al*., 2011).

Out of the 279 rice landraces, most of accessions contained from 0 to 2 different resistant genes. Only four accessions possessed a maximum of 6 resistance genes, 5 genes in three accessions, and 4 genes in four accessions (Fig. 2B). Of the 11 accessions including 4, 5, 6 resistance genes, 4 accessions (IT009221, IT006538, IT009118, and K026194) were also found resistant (≤ 1.0 score on a 0-to-9 scale) in the nursery tests of both 2010 and 2011 (Fig. 2C). We then further surveyed the distribution of blast resistant genes of the 11 selected accessions in the field screens. Among these accessions, all accessions showed the positive bands to *Pit* on chromosome 1. Hayashi *et al*. (2006) reported that *Pit* gene was a major resistance gene on chromosome 1, *indica* rice variety K59, and confer essential race-specific resistance against the blast disease. The other genes also showed

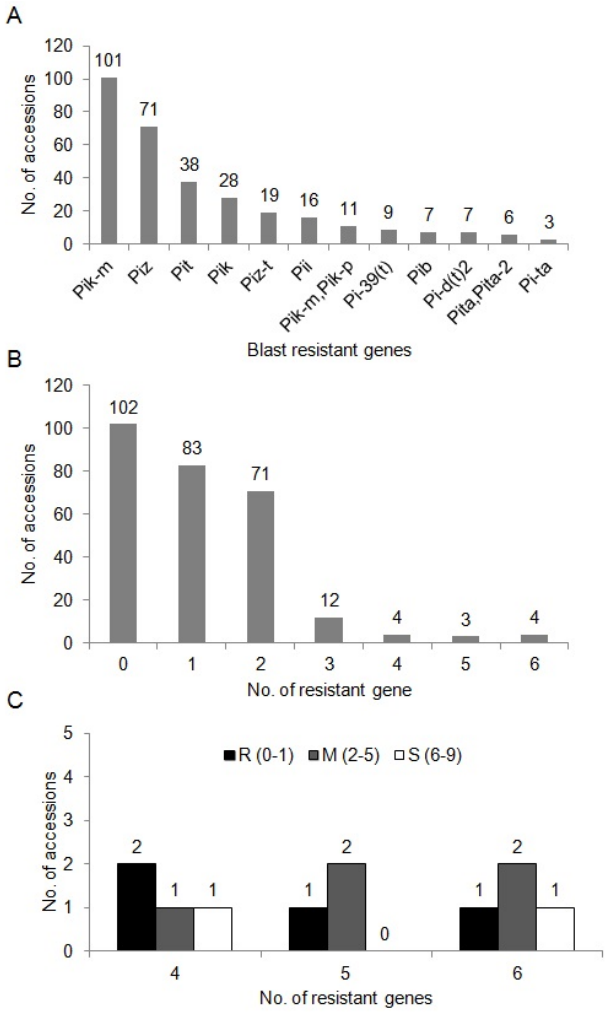

Fig. 2. Frequency distribution of blast resistant genes among all 279 accessions (A) and the frequency of rice accessions having different number of blast resistance genes (B). The degree of blast resistance in 11 accessions including four to six different blast resistant genes (C).

incompatible patterns of PCR amplification between positive and negative markers as blast resistance and susceptible (Fig. 3 and Table 3). The three genes such as *Piz*, *Pik,* and *Pi5*, were observed as negative band patterns (0%) with positive markers in eleven rice blast resistant accessions selected in field tests. Also, among 279 landraces, the *Pik-m* was the most amplified gene, but this gene amplified in only 2 accessions of 11 the resistant accessions selected in the bioassay to rice blast. Thus, the determination of whether resistance could not be completely identified in our present experiments as compared with the field screen tests. We suggested that the 11 selected accessions revealed resistance to blast disease by other genes of a large amount of blast resistant genes.

Genetic statistics and similarity in Korean landraces

The responses to blast among 279 landrace accessions were classified to three types, which are resistant, moderate and susceptible accessions based on nursery test in same regions during 2010 and 2011 and *in vitro* inoculation. We further analyzed the genetic grouping pattern, genetic variability and differences in all landraces using genome-wide SSR markers for compare with the three types clustered on field tests. We obtained the results that the landrace accessions were showed a substantial degree of genetic differentiations. The principal coordinate analysis (PCoA) described the separation of the populations among the landrace accessions (Fig. 4) and indicated the degree of genetic similarity among them. The pattern of groupings revealed two major groups and was the same as the results of blast nursery tests and *in vitro* inoculation. The 11 selected resistant accessions against blast disease in nursery tests are formed from the other group, and some moderate blast resistant accessions were located near resistant clade. The remaining moderate and susceptible accessions were not classified. The result indicated that the entire 279 accessions were distinctly two grouped according to the degree of blast disease incidence (Fig. 4). Table 4 summarizes the average number of alleles (N_A) and polymorphic information content (PIC) for each locus in the 297 rice landrace accessions using genome-wide 64 SSR primer pairs. By comparing genetic diversities with the parameter PIC value among the resistant, total and susceptible landrace on chromosomes (Fig. 5), we found that comparison of PIC values reduced genetic variability in RM580 on chromosome 1, RM527 on chromosome 6, and RM3533 and RM 215 on chromosome 9, while increased genetic variability RM174 on chromosome 2, RM135 on chromosome 3, RM6629 and RM16427 on chromosome 4, RM197 on chromosome 6, and RM519 on chromosome 12 in the resistant accessions contrary to total and susceptible groups. Although it is differ from mean PIC values between resistant and susceptible landraces, these regions might be linked to resistance alleles. Identifying flanking DNA markers from these regions would be yielded high level of selection accuracy for resistance.

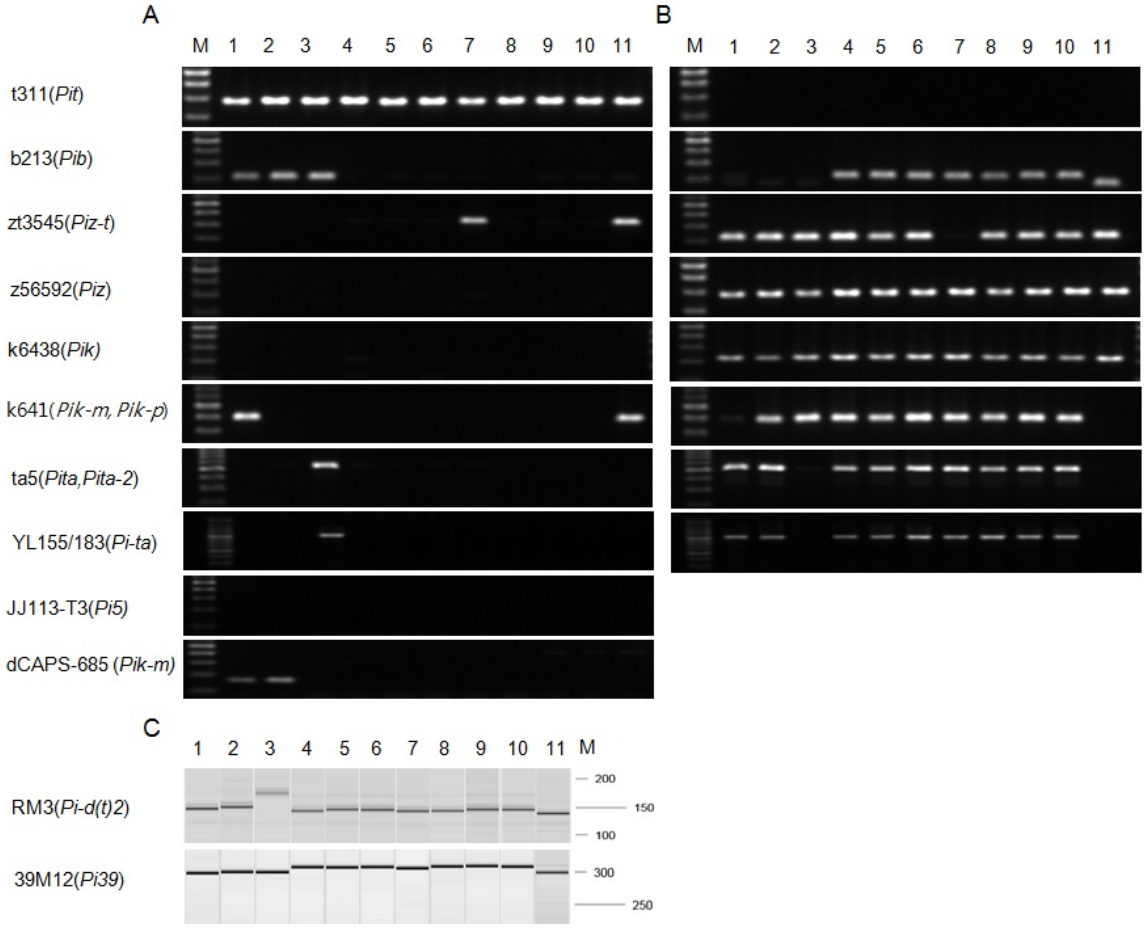

Fig. 3. PCR amplification patterns of twelve markers that discriminate each of the 12 blast resistance genes in 11 the selected resistant landraces. A and C indicates the amplification products obtained with blast resistance specific primer on gel electrophoresis and QIAxcel capillary gel electrophoresis, respectively. B indicates the amplification products obtained with blast susceptible specific primer. Lane 1-11 is IT006538, IT009118, IT009221, IT010345, IT010480, IT010555, IT010565, IT010577, IT010721, IT010726 and K026194.

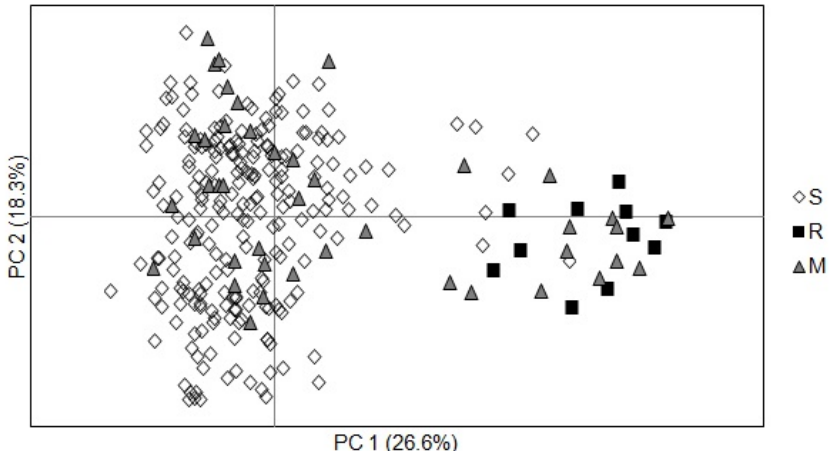

Fig. 4. A scatter of principal coordinate analysis (PCoA) of the 279 rice landraces based on genetic distance estimates. S - susceptible, R - resistant and M - moderate in nursery tests during both 2010 and 2011.

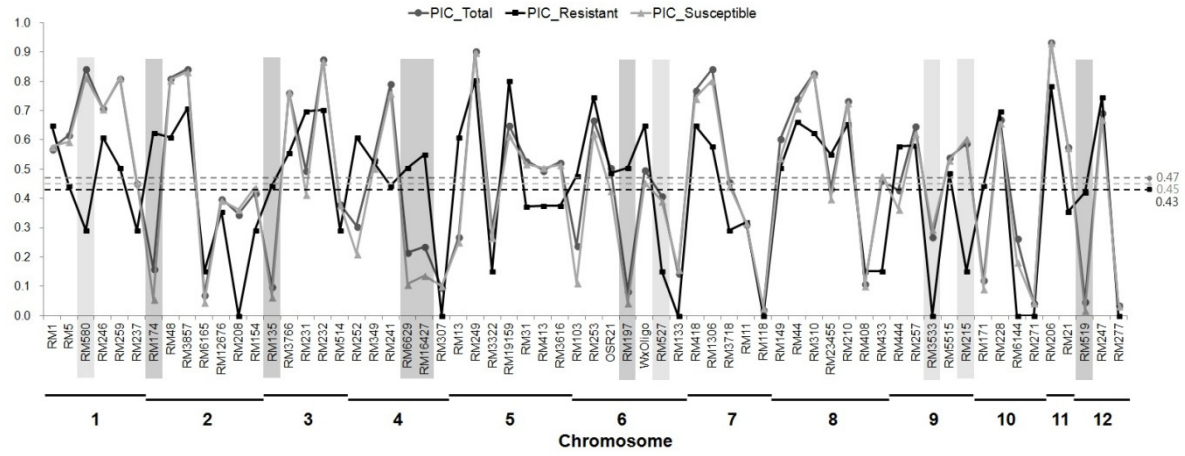

Fig. 5. PIC distribution of SSR loci among resistant, susceptible and total landraces on chromosomes 1-12. Dark gray curve shows PIC changes in total accessions, light gray curve in the susceptible and black curve shows changes in the resistant landraces. The broken line represents average PIC value for all SSR loci in each color landraces. Dark gray box - increased the PIC values and light gray box - decreased in resistant accessions contrary to total and susceptible accessions.

Discussion

Rice blast, which is *Magaporhe grisea*, is one of the most limiting serious factors for rice production throughout rice worldwide. For the last few decades, genetic studies of resistance to rice blast have been conducted and rice geneticists and breeders have tried to collect new resources of resistant germplasm to develop durable resistant cultivars accumulating several blast resistance genes. Rice landraces have been recognized as valuable genetic resources for improving resistance level of modern rice cultivars against biotic disease (Villa *et al*., 2006; Kobayashi *et al*., 2006). The present study was performed to get the blast resistance degree of Korean rice landraces through field nursery tests and *in vitro* inoculation and to investigate the distribution of blast resistant genes using 12 major genes of blast resistance. In this study, 279 landrace accessions were used for an initial screening under blast nursery condition for 2010 and 2011 at same region in Korea, and then were checked blast resistance through *in vitro* inoculation. Based on the scores for the degree of blast disease incidence in the blast nursery test ranged from 0 (no lesions) to 9 (death of all leaves), we selected the resistant accessions for two years at field. Some landrace accessions showed moderate resistant to rice blast, and most of landraces revealed susceptible reactions in the two tested ways. Finally, 11 resistant accessions were selected at field and *in vitro* system for two years. The selected landrace accessions may be an important resource for the improvement of blast resistance and specific agronomic traits. Landraces are valuable genetic resources, because they contain huge genetic variability which can be used to complement and broaden the gene pool of advanced genotypes (Kobayashi *et al*., 2006).

For several decades, several genes of blast resistance were found that are effectively used to control rice blast disease in rice breeding and genetic studies (Chen *et al*., 2005; Ballini *et al*., 2008; Koide *et al*., 2009; McCouch *et al*., 1994). However, blast resistance from the Korean rice landraces has not been found, in spite of the fact that several genes of blast resistance, such as *Pik-p, Pib, Pi-d(t)2, Piz, Pit, Pik-m, Pita/Pita-2, Pik, Pi39* and *Piz-t,* have been identified. We analyzed the distribution of resistant genes, genetic variability and differences using 12 resistant (R) genes markers and 64 genome-wide microsatellite markers in the selected resistance and susceptible accessions. The results revealed that 101 (36.2%) and 71 (25.4%) accessions show the positive reactions of *Pik-m* and *Piz* genes in the tested rice germplasm, respectively. The previous studies described that the *Pik-m* of three *Pik* locus on chromosome 11 confer high and stable resistance to many Chinese rice blast isolates (Zhai *et al*., 2011) and *Piz* gene has been used for conferring blast resistance to Japanese cultivars, because their importance was emphasized by Hayashi *et al*. (2004) in rice breeding in Japan. Nevertheless, these genes

were not amplified in all the resistant accessions selected in the nursery test to rice blast, with the exception of two accessions. These results did not consist of blast nursery screening and *in vitro* inoculation in this study. Thus, we further confirmed the distribution of blast resistant gens in the 11 accessions selected in the field screens for compare the degree of genetic resistance with the result, which is bioassay in the nursery tests. Out of the 279 rice landraces, eleven accessions possessed different resistance genes from 4 to a maximum of 6 genes. Four (IT009221, IT006538, IT009118, and K026194) of those accessions showed resistance ($\leq$ 1.0 score on a 0-to-9 scale) in the nursery tests during both years.

Eleven accessions showed the positive reactions to *Pit* gene, which is a major resistance gene on chromosome 1 and confers essential race-specific resistance against the blast disease (Hayashi *et al.*, 2006). The three genes, *Piz*, *Pik,* and *Pi5* were not identified (0%) and the other genes were also few amplified in eleven resistant accessions, but these genes revealed incompatible patterns of PCR amplification between positive and negative markers as blast resistance and susceptible. In brief, genotypes had different reactions against blast diseases in the phenotypic part of nursery tests. We suggested that the 11 selected accessions showed resistance to blast disease by other genes of a number of blast resistant genes.

For surveying this assumption, we used genome-wide SSR markers that have closely been located with resistance genes. To evaluate genetic variability among tested accessions, the landraces were profiled using genome-wide 64 SSR primer pairs. The PCoA was performed to examine further the genetic similarity among landrace accessions on the basis of the SSR data. The results indicated that blast resistance accessions formed the divergent cluster from the main cluster and some moderate blast resistant accessions were located near resistant cluster. By comparing genetic diversities with the parameter PIC value among the resistant, total and susceptible landrace on chromosomes, we found that PIC values reduced genetic variability in four SSR markers on chromosome 1, 6, and 9, while increased genetic variability in six markers on hromosome 2, 3, 4, 6, and 12 in the resistant accessions contrary to total and susceptible groups. Mapping studies of blast resistance genes have been carried out and major blast resistance genes have been identified and mapped using molecular markers. Many of these *Pi* genes are clustered on chromosomes 6, 11 and 12 (Kinoshita, 1995). These regions might be linked to resistance alleles. This result suggested that genetic differentiation between them might not be on a genome-wide scale, but rather on some selected loci or in some genomic region. These suggested that other 5 genes in flanking region of these 6 genes have an influence on rice blast resistance. Using fine mapping approaches, we might be able to demonstrate that five genes in these regions would be conferred broad-spectrum resistance of the landraces with various gene combinations.

The evaluation results of blast nursery and isolate inoculation test, and distribution of blast resistance genes in rice landrace accessions will help in breeding of blast resistant varieties. The results of present study are very useful for further rice improvement and for stable field resistance to blast races in Korean *japonica* cultivars using landraces accessions. Utilization of local landraces in breeding program may be the good way of genetic resources conservation. In our present study, we were not able to confirm the relationship between the amplification of resistance genes and the reaction of blast resistance, and also the determination of whether resistance could not be completely identified in our present experiments. In this regard, The fine mapping approaches based on the association between putative these genes linked to the blast resistance and field resistance data will be necessary in future experiments to confirm relationship between molecular markers and blast resistance in Korea landrace accessions.

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